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13. ABSTRACT *(Maximum 200 Words)*
Novel anti-tumor strategies are required for breast cancer. We hypothesize that immunotherapy used in a minimal residual disease setting, such as can be obtained following stem cell transplantation, may prevent relapse of disease. Natural killer (NK) cells reconstitute the bone marrow beginning 3-4 weeks following an autologous transplant. We determined that NK cells can be activated with exogenous interleukin-2 (IL-2) to kill breast cancer targets. In order to improve and develop new strategies of immunotherapy, we investigated the mechanisms of NK cell recognition and lysis of breast cancer targets. We found multiple mechanisms to be involved, including β2 integrins, CD2, and LFA-2 and Herceptin antibody dependent cytotoxicity (ADCC). We have further investigated our ability to maximize the activation of NK cells by IL-2 in collaborative laboratory studies in the context of clinical trials. We have demonstrated IL-2 administration during stem cell mobilization enhances the immunologic potential of the graft, and that we can markedly enhance the NK lytic activity towards breast cancer targets in the post-autologous transplant period by subcutaneous IL-2 administration followed by either IL-2 activated lymphocyte infusion or bolus IL-2. A clinical trial studying our ability to translate a laboratory finding of IL-2/Herceptin ADCC into patient treatment is ongoing.

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Burns LJ, Weisdorf DJ, DeFor TE, Ogle KM, Hammer C, and Miller JS. Enhancement of the anti-tumor activity of a peripheral blood progenitor cell graft by mobilization with IL-2 plus G-CSF in patients with advanced breast cancer. Exp Hematol 28(1):96-103,2000.

Burns LJ, Weisdorf, DJ, DeFor TE, Vesole DH, Repka TL, Blazar BR, Burger SC, Panoskaltsis-Mortari A, Taylor C, Zhang M-J, Miller JS. IL-2 based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: A phase I/II trial. Submitted, Biol Blood Marrow Transplant.

INTRODUCTION

Novel anti-tumor strategies are clearly needed for breast cancer. We hypothesize that immunotherapy used in a minimal residual disease state, such as can be obtained following stem cell transplantation, may serve as noncross-resistant therapy and thus prevent relapse. Although NK cells are among the first immune effectors to reconstitute after stem cell transplantation, resting NK cells do not exhibit activity against breast cancer targets until they are activated with exogenous IL-2. A net balance of positive and negative signals determines whether or not NK cells kill a tumor target. To improve current immunotherapy, we have investigated the mechanisms of NK cell recognition and lysis of breast cancer targets.

Following the first two years of this research project, we demonstrated that multiple mechanisms are involved in IL-2 activated NK killing of breast cancer targets, including β2 integrins, CD2, and LFA-3 (CD58) mediated antibody-dependent cytotoxicity (ADCC). In addition, unlike CD58 antibody-mediated ADCC, Trastuzumab (Herceptin) ADCC was minimally affected by blocking antibodies to CD2 or ICAM-1/CD18, suggesting a different mechanism of action. These results were published in Experimental Hematology in 1999. A copy of the manuscript is included in the appendix.

In conjunction with in vitro laboratory studies of IL-2 activated NK cell killing of breast cancer targets, in the second and third years of this project we initiated and completed two clinical trials incorporating IL-2 into the autologous transplantation setting.

The first trial, exploring the combination of IL-2 + G-CSF in stem cell mobilization, was published last year in Experimental Hematology. A copy of the manuscript is included in the appendix. A second trial incorporated IL-2 in the posttransplant setting. This trial included both patients with breast cancer and patients with lymphoma. During this past year (final year of project) the data was analyzed, and the manuscript has been submitted to Biology of Blood and Marrow Transplantation.

At the end of year three of this project we initiated two new clinical trials. Progress made during the past year in these trials is reported here.

BODY

Results obtained during the first two years of this project are summarized below. They met the first two technical objectives as outlined in the original proposal, and results have been published. Please see the appended manuscript, **Cooley S, Burns LJ, Repka T, Miller JS. Natural killer cell cytotoxicity of breast cancer targets is enhanced by two distinct mechanisms of antibody-dependent cellular cytotoxicity against LFA-3 and HER2/neu.** *Exp Hematol* **27:1533-41, 1999**, for the figures referred to below.

Technical Objective 1: Determine the molecular recognition of ICAM-1 constitutive and cytokine induced expression by breast cancer cells.

We originally hypothesized that sensitivity to lysis by IL-2 activated NK cells would directly correlate with relative expression of ICAM-1 on targets. The original grant proposal then focused on determining the mechanism of regulation of ICAM-1 expression at the molecular level (Technical Objective 1, Tasks 1-3). However, our final results did not support this premise (see Figure 2, page 20). There was no correlation between surface expression of ICAM-1 and target sensitivity to NK cell lysis, and induction of ICAM-1 on targets by cytokines failed to make them more susceptible to lysis (Task 4). As our data did not support our original premise, as reported in previous annual reports we did not pursue identification of cis sequences or protein factors regulating ICAM-1 expression; instead, we elected to proceed directly to explore mechanisms of NK killing involving other potential recognition molecules.

Technical Objective 2: Identify recognition molecules other than ICAM-1 that are important in ANK mediated lysis of breast cancer cells.

Results detailed below show that multiple mechanisms are involved in NK cell lysis of breast cancer targets, that none of the targets are inherently resistant to killing, and that two distinct mechanisms of ADCC can target immunotherapy to breast cancer cells.

Role of CD2/LFA-3 interactions in NK cell killing of breast cancer targets
The interaction of CD2 on NK cells with its ligand, CD58 (LFA-3) on breast cancer cells was investigated. CD2 antibody did not significantly effect a change in specific lysis. In contrast, addition of CD58 antibody (AICD58) to targets consistently increased killing of breast cancer targets MB-231, BT-20 and SKBR-3. Addition of the CD58 antibody alone to targets without effectors did not result in lysis, lending further support to the hypothesis that CD58 antibody may function through antibody dependent cellular cytotoxicity (ADCC).

Antibodies against CD58 mediate ADCC
Breast cancer cell lines were phenotyped for surface expression of CD58 (Table 1, page 20). All targets were positive for CD58. Consistent with ADCC, the CD58 (AICD58) antibody effects were independent of IL-2 activation and NK cell CD16 (FcRγIII) was required in the process (Figure 3,page 19 and Figure 4, page 20). We used unique differences between mature NK cells and those derived from long-term cultures of marrow progenitors to generate NK cells that were CD16 negative. We showed that these cells exhibit characteristic lysis of K562 targets demonstrating that their lytic machinery is intact. The failure of the CD58 (AICD58) antibody to enhance killing by the marrow progenitor-derived NK cells demonstrates a requirement for CD16.

Anti-CD-58-mediated ADCC is clone specific, as another CD58 clone (BRIC-5) resulted in no difference in lysis of breast cancer targets by IL-2 activated NK cells. As both CD58 antibodies were isotype IgG2a, the inability of clone BRIC-5 to mediate ADCC may be due to epitope specificity or to some characteristic of tertiary structure (Figure 3).

Trastuzumab (Herceptin) mediates ADCC through a different mechanism
If the CD58 antibody was mediating classic ADCC by signaling through FcRγIII, the significant blocking effect of CD2 would remain unexplained. To further explore this finding, we tested another antibody that mediates ADCC. Herceptin is a humanized antibody against HER/neu2 which has been engineered by inserting the complementary

determining regions of a murine antibody (clone 4D5) into the framework of a consensus human IgG1. Breast cancer cell lines were phenotyped for surface expression of HER2/neu (Table 1, page 20). The HER2/neu murine antibody (clone 2G11, IgG1) did not mediate ADCC. In contrast, Herceptin added to normal CD56+/CD3- NK cells significantly enhanced killing of all breast cancer targets except for MDA-MB-231, the target with the lowest HER2/neu expression (Figure 5, page 22). Titration experiments with the Herceptin antibody and the SKBR-3 target, the target with the highest expression of HER2/neu, showed enhanced lysis down to an antibody concentration of 0.01 ug/mL (n=2), which was the concentration used in subsequent ADCC blocking experiments. Marrow-derived CD16- NK cells did not augment killing of SKBR-3 targets in the presence of Herceptin. Similar to CD58 (AICD58) ADCC, Herceptin augmented killing by resting blood NK cells was also FcRγIII (CD16) dependent as shown using blocking antibodies (see Figure 6). In contrast to CD58 (AICD58) ADCC, which was decreased by nearly 50% by CD2 or ICAM-1/CD18, these same blocking antibodies had less of an effect on Herceptin ADCC. Whereas blocking both CD2 and ICAM-1/CD18 completely abrogated CD58 (AICD58) ADCC, ADCC with Herceptin was only slightly blocked with the same combination of antibodies.

CD58-mediated ADCC but not Herceptin-mediated ADCC is dependent on CD2
Although both antibodies [CD58(AICD58) and Herceptin] result in CD16-dependent killing, blocking experiments suggest different interactions with accessory receptor/ligand pairs. CD58 (AICD58)-mediated ADCC appears to be CD2 dependent, whereas Herceptin ADCC is minimally affected by blocking CD2. To further test this, we used a subset of NK cells that is CD56 and CD16 positive but CD2 negative. This subset, which generally comprises 10 to 40% of normal blood NK cells, was purified by flow cytometry (Figure 7A, page 23). Secondary staining of CD56+/CD2- sorted NK cells showed that greater than 80% expressed CD16. CD56+/CD16+/CD2- NK cells were still able to augment target lysis of Herceptin-treated SKBR-3 targets, which suggests a CD2-independent mechanism of ADCC signaling through CD16. In contrast CD56+/CD16+/CD2- NK cells did not lyse CD58 (AICD58) antibody-treated BT-20 targets, which confirms the CD2 dependence of this ADCC and the lack of triggering through CD16 alone (Figure 7B, page 23).

Technical objective 3: Transcription factors mediating constitutive and cytokine induced expression of HLA class I genes. This objective was not pursued after the data for Objectives 1 and 2 were obtained. At that time in the research project, we elected to focus on translation of the above laboratory findings into the clinical setting in the context of clinical trials with laboratory correlates. A major objective of these trials was to determine if the IL-2 activation of NK cells with enhanced cytotoxicity towards breast cancer cell lines seen in our in vitro studies would translate into in vivo activation of NK cells. Secondary objectives included the safety and efficacy of this approach.

Clinical Trials performed as translational studies of this basic research:

1. Mobilization of peripheral blood stem cells with IL-2 + G-CSF

As we had shown that IL-2-activated NK cells mediate significant cytotoxicity against breast cancer targets in vitro (see Figure 1, *Exp Hematol*, 1999), we hypothesized that mobilization of stem cells with IL-2 and granulocyte colony-stimulating factor (G-CSF) could enhance the anti-tumor activity of the graft in breast cancer patients receiving an autograft. We determined the dose-limiting toxicity and maximum tolerated dose of subcutaneous IL-2 given with G-CSF for peripheral blood stem cell mobilization, the ability of IL-2 + G-CSF mobilized stem cells to reconstitute hematopoiesis, and the in vitro immunologic function of the graft in patients with advanced breast cancer. Results of this clinical trial were published this year. **A copy of the manuscript is included in the appendix (Burns LJ, Weisdorf DJ, DeFor TE, Repka TL, Ogle KM, Hummer C, Miller JS. Enhancement of the anti-tumor activity of a peripheral blood progenitor cell graft by mobilization with interleukin 2 plus granulocyte colony-stimulating factor in patients with advanced breast cancer.** ***Exp Hematol*** **28:96-103, 2000).** Please see this manuscript for the figures referred to below.

Clinical tolerability of IL-2 mobilization
Forty-three consecutive women 18 to 65 years of age with chemosensitive stage IIIA, IIIB, or metastatic breast cancer were enrolled between May 1996 and January 1998 (Table 1, page 27) and received IL-2 + G-CSF for stem cell mobilization. In addition, 15 patients with similar disease characteristics were treated with G-CSF alone for mobilization. IL-2 (provided by Chiron, Emeryville, CA) was administered subcutaneously days 1-14 in a dose-escalated manner (Table 2, page 27). G-CSF 5 ug/kg/day was administered subcutaneously days 8-14 of the mobilization regimen, with apheresis on days 13-15. The minimum required number of CD34+ cells was 1.5 x

10^6/kg. Dose limiting toxicity of IL-2 was determined to be 2.25 x 10^6 IU/m2/day; the maximum tolerated dose 1.75 x 10^6 IU/m2/day.

CD34+ content of stem cell collections
The minimum number of CD34+ cells were achieved following three initial aphereses in 52% of 42 patients undergoing collections after priming with IL-2 + G-CSF (Table 3, page 29). Of the 15 control patients who were primed with G-CSF alone, 93% achieved the target with three aphereses. The need for additional mobilization in patients who received IL-2 versus control patients could not be attributed to differences in prior therapy between the groups of patients. The mechanism of how IL-2 decreases progenitor cell mobilization is not known.

Engraftment of IL-2 mobilized stem cells
Platelet recovery as well as neutrophil recovery was similar in both groups of patients.

Graft phenotype and cytolytic function
Blood mononuclear cells (MNCs) were studied from patients prior to mobilization (baseline), after 7 days of IL-2 but before initiation of G-CSF and from the stem cell product. Cells were tested in cytotoxicity assays, without further exogenous activation, against the K562 tumor target as a measure of NK cell function. Addition of IL-2 to G-CSF mobilization reversed G-CSF induced NK cell suppression (Figure 1, page 30). It also increased IL-2 activation and cytotoxicity of MNCs against breast cancer targets (Figure 2, page 30). Addition of IL-2 to G-CSF mobilization also increased the number of NK cells and activated T cells in the peripheral blood progenitor product (Figure 3).

Summary
We concluded that subcutaneous IL-2 can be given safely in conjunction with G-CSF to mobilize peripheral blood stem cells. Our results demonstrate that IL-2 + G-CSF may be an effective way to enhance the number and function of anti-tumor effector cells within an autograft without compromising hematologic recovery. A major limitation to the use of IL-2 for priming is the decrease in number of CD34+ cells mobilized, a limitation that theoretically may be overcome by an increased dose of G-CSF and/or timing of stem cell collections. In addition, the duration of the enhanced graft-vs-tumor effect mediated by the IL-2 + G-CSF mobilized graft is short, signifying the need for additional posttransplant immunotherapy to maintain and enhance anti-tumor effector cell function.

2. Posttransplant immunotherapy
As the duration of the enhanced graft-vs-tumor effect mediated by an IL-2 + G-CSF mobilized graft is short, additional posttransplant immunotherapy will be required to maintain and enhance any anti-tumor effector cell function of the graft. Therefore, we have continued our efforts in exploring posttransplant immunotherapy as effective treatment in the prevention of disease relapse.

Post-transplant clinical trial with IL-2: We have completed a trial of post-transplant IL-2 in 22 patients with metastatic breast cancer (patients with lymphoma were also enrolled onto this study). The objectives of this trial were to determine the maximum tolerated dose of subcutaneous IL-2 that can safely be given following hematopoietic recovery from autologous transplantation, as well as the safety and immune activating effects of intravenous infusion of either ex vivo IL-2 activated NK cells (part I of the study) or IL-2 boluses (part II of the study) (Figure 1, page 65). A manuscript detailing the results of this clinical trial has been submitted to *Biology of Blood and Marrow Transplantation.* **A copy of this manuscript, Burns LJ, Weisdorf, DJ, DeFor TE, Vesole DH, Repka TL, Blazar BR, Burger SC, Panoskaltsis-Mortari A, Taylor C, Zhang M-J, Miller JS. IL-2 based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: A phase I/II trial,**, is included in the appendix. Please see this manuscript for the figures referred to below.

Clinical tolerability of subcutaneous IL-2 therapy
Thirty-four patients (metastatic breast cancer, n=14) were enrolled to 4 dose levels of IL-2 (Table 2, page 58). All but 2 patients enrolled at the dose levels of 0.25 to 1.25 x 10^6 IU/m^2/day were able to dose escalate by one dose level at day 14 of IL-2 treatment. One patient enrolled at 0.75 x 10^6 IU/m^2/day did not dose escalate secondary to thrombocytopenia (decline from 201,000/μL to 139,000/μL after 14 days of therapy); one patient enrolled at 1.25 x 10^6 IU/m^2/day did not dose escalate because of fatigue and nausea. One patient was removed from study after completing 22 days of IL-2 at 0.25 x 10^6 IU/m^2/day after developing a skin abscess at an injection site. Toxicities

were otherwise mild, including Grade I fatigue, nausea, rash, cough, fever, myalgias, and sweats. In addition, the majority of patients developed transient local skin induration and erythema around the subcutaneous injection sites, typically 1-2 cm in diameter.

A total of 25 patients began IL-2 at 1.75 x 10^6 IU/m^2/day. Ten (40%) of patients were unable to have their dose escalated at day 14 due to side effects, and 7 of these patients ultimately were removed from study. Toxicities included: severe swelling of the tongue and face (n=1), thrombocytopenia (n=2), nausea and vomiting (n=1), intolerable fatigue (n=5), edema (n=1), and bacterial infection (n=1). Eighteen (72%) of the 25 patients who began IL-2 at 1.75 x 10^6 IU/m^2/day received $\geq$ 95% of the total number of planned injections. Fifteen (60%) of the 25 patients were able to dose escalate to 2.25 x 10^6 IU/m^2/day on day 14. Of these, 13 were able to remain on the higher dose for the duration of treatment, whereas the other 2 patients required a subsequent decrease back to the 1.75 x 10^6 IU/m^2/day dose level because of either anemia or thrombocytopenia. No patient required hospitalization, and all toxicity resolved within one week of discontinuation of IL-2. The MTD was 1.75 x 10^6 IU/m^2/day; therefore, no patients were enrolled at a starting dose of 2.25 x 10^6 IU/m^2/day.

Clinical tolerability of ex vivo IL-2 activated cell infusions
All patients who received subcutaneous IL-2 at less than the MTD (0.25 - 1.25 x 10^6 IU/m^2/day) received a cell dose of 4.0 x 10^7 cells/kg (Table 3, page 59). Grade I toxicities observed infrequently at this dose level included fever and chills at time of the infusion. At the MTD of subcutaneous IL-2, 1.75 x 10^6 IU/m^2/day, 4 patients received a cell dose of 4.0 x 10^7 cells/kg (1 patient underwent one apheresis, the remaining patients two aphereses); 6 patients received 8.0 x 10^7 cells/kg (2 patients underwent one apheresis, the remaining 4 patients two aphereses);10 patients underwent two lymphaphereses with reinfusion of the full product. Grade I-II toxicities included wheezing (n=1), mild fever (37.1-38.0^0 C) (n=4), chills (n=4), transient decrease in oxygen saturation to 88% during chills (n=1), and transient decrease of systolic blood pressure to 80-85 mm Hg (n=1). No patient required hospitalization. Two patients were removed from study after the first apheresis (1 for facial swelling, 1 for a bacterial infection as above). A third patient declined the second apheresis secondary to difficulties with venous access but remained on subcutaneous IL-2 for the duration of the study.

Clinical tolerability of IL-2 boluses
All 23 patients received subcutaneous IL-2 at the MTD of 1.75 x 10^6 IU/m^2/day. Three patients made up the first cohort, and all 3 received 42 days of subcutaneous IL-2 injections and both planned IV IL-2 boluses of 2 x 10^6 IU/m^2 (Table 4, page 60). Six (50%) of the 12 patients enrolled at the 4 x 10^6 IU/m^2 dose level received both IV boluses. Both boluses were not infused due to thrombocytopenia (n=2), Clostridium difficile infection (n=1), rash and insomnia (n=1), and neutropenia (n=1). Of the 12 patients, 8 received all 41 - 42 doses of subcutaneous IL-2; 4 patients received $\leq$23 days of subcutaneous IL-2. Grade I toxicities occurring at the IL-2 bolus dose of 4 x 10^6 IU/m^2 included fever (n=2), chills (n=3), and shortness of breath (n=1). Orthostatic hypotension occurred in 2 patients - one patient responded to intravenous normal saline, the other had spontaneous resolution of symptoms. All 8 patients enrolled at the highest dose of bolus IL-2, 6 x 10^6 IU/m^2, received all 42 days of subcutaneous IL-2 injections and both infusions. Grade II toxicities of fever and chills were noted in all patients during the bolus infusions. Three patients experienced a $\geq$10 mmHg decline in systolic blood pressure and required infusion of normal saline for blood pressure support.

Immune activation after IL-2 therapy
Laboratory tests were performed every two weeks to monitor immune activation. There was a significant increase in the total white blood cell count, peaking at day 14 and after each activated cell infusion or IL-2 bolus infusion, analogous to our previously reported findings with post-transplant subcutaneous IL-2 .The increase in total white blood cell count was primarily due to an increase in the number of circulating lymphocytes and eosinophils; however, the absolute number of circulating monocytes did not change with IL-2 therapy.

The lymphocyte increase included a 10-fold increase in circulating NK cells which was sustained throughout the IL-2 treatment period. Less than 2% of CD56+ cells expressed CD3 at any time point. In contrast to the increase in NK cells, the absolute number of T-cells remained fairly stable throughout IL-2 administration. Again, this was consistent with our previous findings.

We tested the lytic function of PBMNC obtained at study entry prior to any subcutaneous IL-2 administration, pre- and post-infusion of IL-2 activated cells, and also tested the *ex vivo* activated product against MCF-7 cells

(Figure 2, page 66). Prior to subcutaneous IL-2, fresh nonactivated PBMNC had no lytic activity (<10%). *In vitro* cytotoxicity was modestly enhanced following 28 days of subcutaneous IL-2. In contrast, cytotoxicity was markedly enhanced for cells incubated overnight with IL-2 (product), and from fresh PBMNC obtained the day post-infusion of the activated cells. The lytic activity of PBMNC obtained from patients after 42 days of subcutaneous IL-2 was similar to that at 28 days. In addition, the enhancement in cytotoxicity following the second IL-2 activated cell infusion on day 42 was as potent as the first (data not shown). This suggests that the enhanced cytolytic function of the IL-2 activated cells was less than 2 weeks in duration.

The lytic function of PBMNC obtained pre-infusion and 1 day post- infusion of IV boluses of IL-2 is shown in Figure 3, page 67. Cytolytic activity after administration of subcutaneous IL-2 alone was similar to that of patients in part I of the study. Post-infusion of IL-2 boluses, cytotoxicity was markedly enhanced to a degree comparable to that seen following infusion of *ex vivo* IL-2 activated cells.

Cytokines induced by IL-2

We determined the circulating cytokine levels of IL-6, IFN-γ, TNF-α, and IL1-β in patients at day 0, day 14, and throughout their course of bolus IL-2 infusions. As shown in Figure 4, page 68, IL-2 boluses transiently increased the levels of all four cytokines, although the increase in the level of IL-1β was minimal. Peak levels of IL-6, IFN-γ, and TNF–α were noted 2 hours after each IL-2 infusion. The peak level for IL1-β occurred 24 hours after infusion; a second peak was not noted after the second IL-2 bolus. There was a dose dependent increase in IL-6 and IFN-γ. Patients who received 4-6 x 10^6 IU/m^2 had a statistically significant greater increase in peak cytokine levels of IL-6 and IFN-γ compared to patients who received 2 x 10^6 IU/m^2 of IL-2.

Clinical outcome following IL-2 immunotherapy: Matched pairs analysis

Thirteen (59%) of the 22 patients with metastatic breast cancer who received ≥ 1.75 x 10^6 IU/m^2/day of subcutaneous IL2 in conjunction with at least one of the planned IL-2 activated cell infusions or IL-2 boluses were able to be matched with control patients from the ABMTR. The other 9 patients had no suitable controls identifiable. For the 13 patients with breast cancer, one patient was matched with 1 control, 3 patients with 2 controls, and 9 patients could be matched with 3 controls from the registry. Patient characteristics are shown in Table 5, page 61. There were either very minimal or no differences between case patients and control patients for all matching characteristics. Disappointingly, we observed no difference in survival or disease-free survival between case and control patients (Table 6, page 62). Although no suggested advantage for the IL-2 based therapy was apparent, the power power to detect a difference in this analysis was low due to the limited patient numbers. Therefore, we feel that no firm conclusions can be drawn regarding the efficacy of this dose and schedule of post-transplant IL-2 based immunotherapy.

Summary

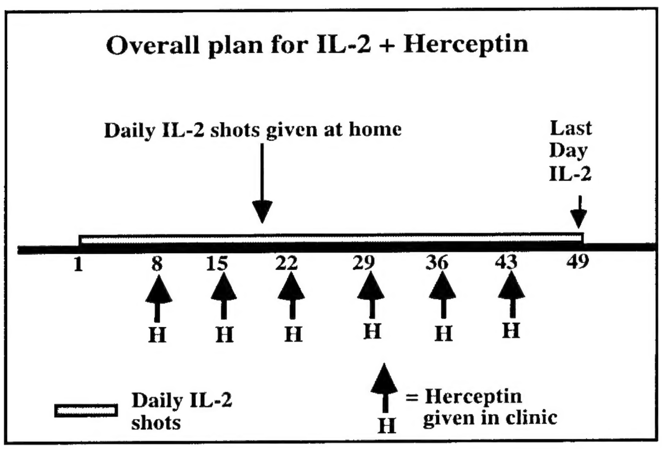
From this clinical trial, we conclude that IL-2 based immunotherapy with either IL-2 activated effector cells or IL-2 boluses is safe, and results in significantly enhanced cytolytic function against breast cancer targets. Unfortunately, given the limited patients for whom matched controls could be found, we cannot draw firm conclusions as to the efficacy of this aproach. However, further enhancement of the potency of IL-2 activated NK cytotoxicity will most likely be required in order to be efficacious. Potential approaches could include a combination of NK cells with tumor-reactive monoclonal antibodies to induce ADCC (see ongoing trial described below), utilization of highly purified NK cells or subsets, combination with chemotherapy or other cytokines such as IL-12, or augmentation by NK cell inhibitory receptor blockade.

While awaiting matuation of the data and analysis for the post-transplant IL-2 trial, we proceeded to test the hypothesis that IL-2 alone may not have significant efficacy in patients with metastatic breast cancer. We obtained study support to investigate IL-2 in combination with other agents:

b. <u>IL-2 + SCF</u>: In 2000 we initiated a post-transplant immunotherapy trial with IL-2 + SCF for patients with metastatic breast cancerr. Correlative in vitro assays of NK, T-cell and dendritic cell function in response to IL-2 + SCF were planned. The trial was submitted and approved by the Institutional Review Board. However, we subsequently had to close the study prior to enrolling any patients with breast cancer, as the study sponsor (Amgen) decided to no longer supply SCF for this indication.

c. Flt-3 ligand: As the SCF trial was not be able to be performed, we instead initiated a trial of Flt-3 ligand as post-transplant immunotherapy in patients undergoing autologous transplantation for metastatic breast cancer. Flt-3 ligand (20 ug/kg) was administered subcutaneously 3 x/week for a total of 21 doses in a phase I trial. Patients were eligible for this study 56-112 days after autologous transplant if they had hematologic recovery and no ongoing therapy related toxicities. Eight patients were treated with Flt3 ligand on this trial. There were no constitutional symptoms related to this immunotherapy and no clinical toxicity or hospitalizations. Monocytes were increased in all patients on therapy. Dendritic cell (DC) numbers determined by 4-color immunophenotype analysis from blood at day 14 showed a median 25-fold [4-52-fold] increase in CD11c+, HLA-DR+, CD14- DC compared to blood obtained prior to initiating therapy. Co-stimulatory molecule expression (CD80 and CD86) on *in vivo* mobilized DC was lower than that found on monocyte derived DC generated *in vitro*. Two of three evaluable patients had an increased ability to stimulate an allogeneic mixed lymphocyte reaction on day 14 of therapy and the third patient was unchanged compared to baseline. There was no significant change in NK cell numbers or cytotoxicity assays against K562 targets throughout the study period suggesting that NK cells are not stimulated *in vivo* by Flt3 ligand as found in the mouse. We concluded that Flt3 ligand could be administered safely early after autologous transplant to increase circulating DCs - a strategy that may be useful alone or in combination with other agents to elicit specific anti-tumor immunity. Unfortunately, the study sponsor (Chiron) notified us this month that they are not going to proceed with drug development of Flt-3, so this trial has now been closed.

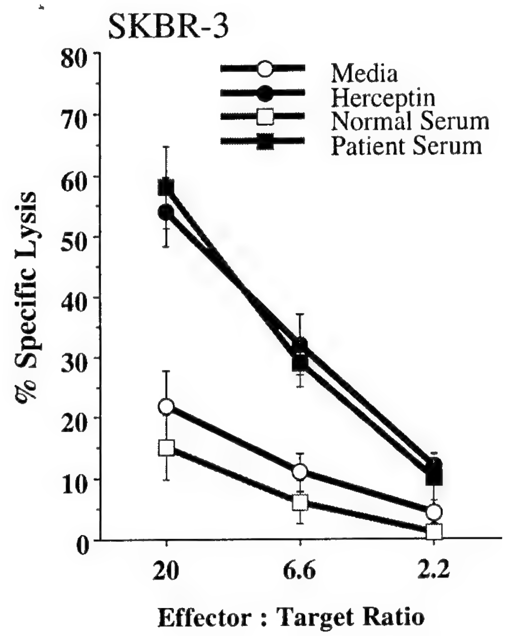
3. NK cell function in response to IL-2 + Herceptin



Based on our subcuatenous IL-2 data showing a 10-fold increase in circulating NK cells and the ADCC we had noted with Herceptin , a phase I clinical trial was performed to test the safety of IL-2 combined with Herceptin. The schema for this therapy is shown to the left. IL-2 was given daily at 1.75 MU/m2/day for 49 days and Herceptin (4 mg/kg load, then 2 mg/kg weekly) for 6 weeks.

Ten women with HER2 positive metastatic breast cancer were enrolled (one patient was re-enrolled in the study after completing the protocol one year earlier). Three patients were not evaluable. Of the 7 evaluable patients, one patient had a partial remission, 4 had stable disease, and 2 had progressive disease. These patients were heavily pretreated, one having relapsed disease following an autologous transplant, and had progressed on Herceptin+/- Chemotherapy regimens. She responded to this Herceptin/IL-2 regimen with near complete resolution of the subcutaneous metastatic lesions on her back. However the treatment duration was short. No unexpected toxicities were seen.

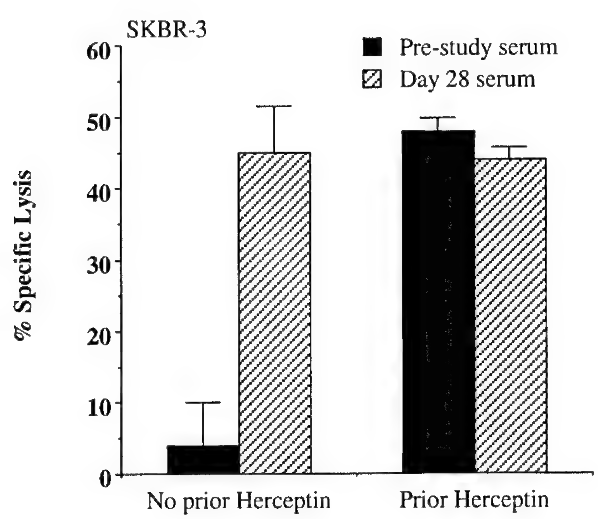
Laboratory Correlates from the Phase I trial. Blood mononuclear cells and serum were collected from patients on study. The absolute number of circulating NK cells and their nonspecific lytic activity against K562 targets was significantly increased on therapy. Herceptin, patient serum or normal human serum did not alter this cytotoxicity. In contrast, 22±6% of HER2/neu overexpressing SKBR-3 targets were lysed by patient cells on therapy (> 28 days) compared to the 1.3±1% pre-study baseline. Specific lysis was further enhanced by the addition of Herceptin (54±6%) or patient serum 7 days after a Herceptin infusion (58±7%) compared to normal human serum (15±5%), which contributed no added activity (Figure). Similar but less augmented killing was seen against a HER2/neu low expressing target (MCF-7).



Peripheral blood mononuclear cells (PBMC) from breast cancer patients were studied in standard 4-hour chromium release cytotoxicity assays against SKBR-3 targets. All patients exhibited a significant increase in circulating NK cells as we have shown previously with these doses of subcutaneous IL-2 (data not shown). Patient PBMC was tested with media alone (Media), exogenous fresh Herceptin added in vitro (Herceptin,1 μg/ml), 25 % normal serum (Normal serum) and 25% patient serum prior to the next Herceptin infusion (Patient Serum).

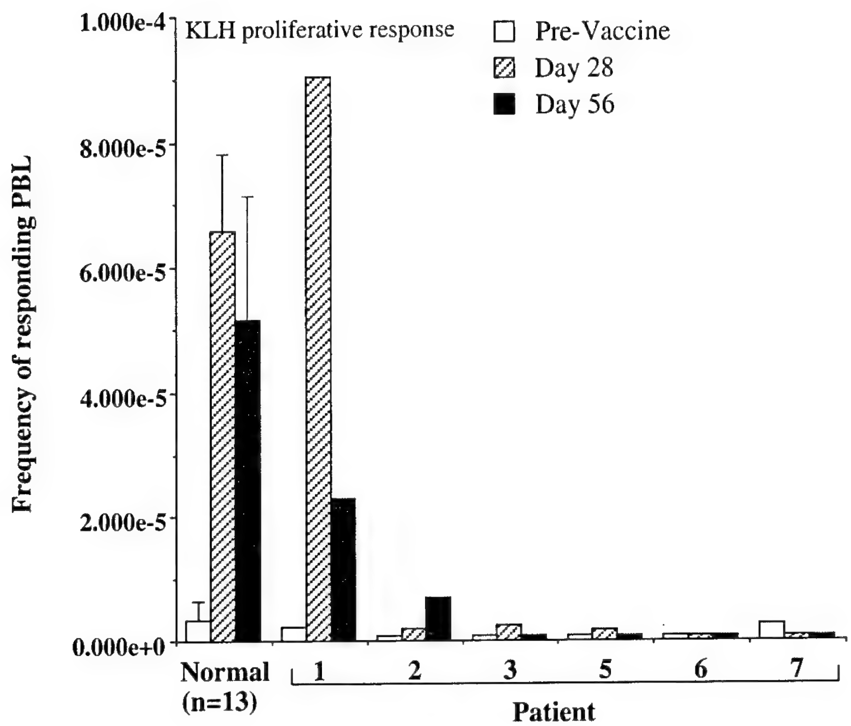
These results show that 1) NK cells can safely be expanded *in vivo* with outpatient therapy, 2) Herceptin augments NK cell killing of breast cancer targets in a HER2/neu specific manner, and 3) trough patient serum contains physiologic levels of Herceptin capable of mediating ADCC. Although not shown here, significantly augmented killing was also seen with low HER2/neu expressing breast cancer cells (MCF-7) but less than with high expressing SKBR-3 targets.

We further studied the serum samples from patients against normal allogeneic NK cells. Mononuclear cells were enriched for NK cells using immunomagnetic beads. Serum from patients prior to study and from samples collected on day 28, prior to the next Herceptin dose (a trough serum), were added to normal NK cells and SKBR-3 targets. Two patterns were seen. In patients who never received Herceptin, there was a marked change in serum ADCC activity on therapy compared to pre-study (see figure below). Surprisingly, in patients 2-8 weeks from a previous course of Herceptin, serum ADCC activity was still maintained. This suggests that the biologic half-life of this activity may last weeks between Herceptin doses and possible longer. This ADCC assay seems to be a good bioassay for measuring ADCC activity. It also supports lengthening the interval from one to two weeks between Herceptin infusions and an even longer interval between patient courses. A phase II study is currently being planned.



4. <u>Vaccination with tetanus toxoid (TT) and Keyhole Limpet Hemocyanin (KLH) to assess antigen specific responses following autologous transplantation.</u>

A vaccine may help to eradicate minimal residual disease. However, even the best vaccine strategy will fail if the immune system is not able to respond appropriately. To test immune integrity, we vaccinated 15 normal volunteers with 1 mg of KLH, a neo-antigen that humans would not have been exposed to previously and 10 received tetanus toxoid (TT), an antigen that all subjects have seen. Proliferation assays were performed in limiting dilutions to allow frequency calculations. The frequency of KLH-specific mononuclear cells increased 20-fold 28 days after a KLH vaccine and 100% of normal subjects responded. As expected, the frequency of TT-specific cells was higher at baseline and increased 3.6-fold after vaccination. Similar responses were seen using ELISPOT readouts for cells producing INF-γ. KLH elicited potent IgM, IgG1, IgG2 and IgG3 specific antibody responses in all normal subjects and TT-specific IgG1 increased as well. We next vaccinated 6 patients (breast cancer, myeloma, CML) 3-19 months after autologous transplant. In marked contrast to normal subjects, only 1 of 6 patients exhibited a KLH proliferative response (see figure below) and 1 of 5 exhibited a TT response. No patient mounted a specific humoral response. The poor response in transplant patients is of major concern if vaccines are to be added early when disease burden is lowest.



KEY RESEARCH ACCOMPLISHMENTS:

- We defined the immunologic mechanisms involved in IL-2 activated NK cell killing of breast cancer targets, with publication of a peer-reviewed manuscript.

- We demonstrated that the anti-tumor activity of a peripheral blood progenitor cell graft can be enhanced by mobilization with IL-2 + G-CSF in patients with advanced breast cancer, with publication of a peer-reviewed manuscript.

- We completed a post-transplant immunotherapy trial of subcutaneous IL-2 plus IL-2 activated cells/IL-2 boluses in patients undergoing transplantation for metastatic breast cancer. We demonstrated that the IL-2 could be safely given with either IL-2 activated cells or IL-2 boluses in the outpatient setting, and that cytolytic activity of NK cells towards breast cancer targets was greatly enhanced by either IL-2 regimen. Disappointedly, a matched pairs analysis failed to suggest a survival advantage for IL-2; however, the number of patients who could be matched with registry controls was a limitation of the analysis.

- We are performing additional clinical trials with correlative in vitro studies of NK cell, B cell, T cell and dendritic function with other immunotherapeutic agents, including the monoclonal antibody, Herceptin, and vaccines.

REPORTABLE OUTCOMES:

1. PUBLICATIONS

Cooley S., Burns LJ, Repka, T, Miller JS. Natural killer cell anti-tumor cytotoxicity against breast cancer targets is mediated by several mechanisms which are further augmented through ADCC by an antibody that recognizes LFA-3. Exp Hematol 27(10):1533-41, 1999.

Burns LJ, Weisdorf DJ, DeFor TE, Ogle KM, Hammer C, and Miller JS. Enhancement of the anti-tumor activity of a peripheral blood progenitor cell graft by mobilization with IL-2 plus G-CSF in patients with advanced breast cancer. Exp Hematol 28(1):96-103,2000.

Burns LJ, Weisdorf, DJ, DeFor TE, Vesole DH, Repka TL, Blazar BR, Burger SC, Panoskaltsis-Mortari A, Taylor C, Zhang M-J, Miller JS. IL-2 based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: A phase I/II trial. Biol Blood Marrow Transplant, submitted August 2001, suggested revisions have been made and are currently being reviewed. When accepted, a preprint will be forwarded.

2. ABSTRACTS/PRESENTATIONS

Burns L, Weisdorf D, Ogle K, Hummer C, Miller JS. Mobilization of a PBPC graft with anti-tumor activity using interleukin-2 (IL-2) plus G-CSF in patients with advanced breast cancer. Blood 1997; 90:593a. Oral presentation at national meeting of the American Society of Hematology.

Miller JS, Weisdorf D, Ogle K, O'Keefe P, Burger SR, Burns L. Outpatient post-autotransplant immunotherapy with IL-2 activated lymphoid cell infusions: More cytotoxicity versus IL-2 alone. Blood 1997:90:382b. Poster presentation at national meeting of the American Society of Hematology.

Burns L, Cooley S, Repka T., Miller J. Natural killer (NK) cytotoxicity of breast cancer targets. Poster presentation at the Department of Defense Era of Hope meeting, Atlanta GA, 2000.

Personnel receiving pay from the research effort: Linda J. Burns, M.D.

CONCLUSIONS

We feel that our work has significantly advanced the mechanisms involved in NK cell cytotoxicity of breast cancer targets. We have successfully translated the results into several clinical trials.

The first two trials focused on IL-2 in the autologous transplant setting for patients with metastatic breast cancer. One of the objectives was to determine if the IL-2 activation of NK cells with resulting enhanced cytotoxicity towards breast cancer cell lines seen in our in vitro studies would translate into in vivo activation of NK cells. We have explored the use of IL-2 as a stem cell mobilizing agent, and as posttransplant immunotherapy. Although IL-2 given in conjunction with G-CSF for mobilization of stem cells indeed enhanced the anti-tumor activity of the graft, an increased number of aphereses were required in order to obtain adequate number of CD34+ cells for transplantation. This limitation might be able to be overcome with variations in dosing of the G-CSF or timing of aphereses. In addition, the enhanced immune function was relatively short lived in the posttransplant time period, pointing to the necessity of posttranplant immunotherapy. The second clinical trial utilized IL-2 in the post-transplant setting. We studied the combination of subcutaneous IL-2 plus either IL-2 activated NK cell infusions or infusions of bolus IL-2. In vitro laboratory correlates confirmed that the activity of activated NK cells was greatly enhanced by ex-vivo incubation of lymphaphersis products with IL-2, or by IL-2 bolus infusions. However, efficacy could not be determined due to limited patient numbers in the matched-pairs analysis. Consideration is being given to an expanded phase II trial.

Meanwhile, we are continuing to explore NK function, B cell and T cell function and dendritic cell function in patients receiving other immunotherapy agents in the non-transplant setting. We are particularly interested in the combination of IL-2 with Herceptin, as our in vitro data demonstrated a significant ADCC effect with Herceptin. A phase I clinical trial has been completed, with demonstration of the safety of this approach as well as confirmation of ADCC mediated cytotoxicity against breast cancer targets. A phase II trial is being planned.

Finally, we are interested in tumor vaccines to more specifically target therapy. There are many questions that need to be answered about how best to manipulate the immune system to enhance vaccine responses. In a clinical protocol, we are testing the immune response of patients recovering from autologous transplantation using two known safe vaccines. Laboratory studies include measurement of IgM and IgG antibodies, and T-cell proliferation before and after the vaccines. Preliminary data reported here suggests that the post-transplant time period may be ineffective for generation of an immune response, and we are now determining antigen specific responses in non-transplanted patients. These studies will provide the basis for immunotherapeutic strategies in future investigations.

APPENDICES

Three manuscripts:

Cooley S., Burns LJ, Repka, T, Miller JS. Natural killer cell anti-tumor cytotoxicity against breast cancer targets is mediated by several mechanisms which are further augmented through ADCC by an antibody that recognizes LFA-3. Exp Hematol 27(10):1533-41, 1999.

Burns LJ, Weisdorf DJ, DeFor TE, Ogle KM, Hammer C, and Miller JS. Enhancement of the anti-tumor activity of a peripheral blood progenitor cell graft by mobilization with IL-2 plus G-CSF in patients with advanced breast cancer. Exp Hematol 28(1):96-103,2000.

Burns LJ, Weisdorf, DJ, DeFor TE, Vesole DH, Repka TL, Blazar BR, Burger SC, Panoskaltsis-Mortari A, Taylor C, Zhang M-J, Miller JS. IL-2 based immunotherapy after autologous transplantation induces immune activation and cytokine release yet does not impact on disease outcomes. Biol Blood Marrow Transplant, submitted August 2001, suggested revisions have been made and are currently being reviewed. When accepted, a preprint will be forwarded.

ELSEVIER

EXPERIMENTAL HEMATOLOGY

Experimental Hematology 27 (1999) 1533–1541

Natural killer cell cytotoxicity of breast cancer targets is enhanced by two distinct mechanisms of antibody-dependent cellular cytotoxicity against LFA-3 and HER2/neu

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Treatment of advanced breast cancer with autologous stem cell transplantation is limited by a high probability of disease relapse. In clinical trials, interleukin 2 (IL-2) alone can expand natural killer (NK) cells in vivo and increase their cytotoxic activity against breast cancer cell lines, but this increase is modest. Understanding the mechanisms that mediate NK cell lysis of breast cancer targets may lead to improvements of current immunotherapy strategies. NK cells from normal donors or patients receiving subcutaneous IL-2 were tested in cytotoxicity assays against five breast cancer cell lines. The role of adhesion molecules and antibodies that interact through Fc receptors on NK cells was explored. NK cell lysis of breast cancer targets is variable and is partially dependent on recognition through ICAM-1 and CD18. While blocking CD2 slightly decreased cytotoxicity, contrary to expectations, an antibody against CD58 (the ligand for CD2), failed to block killing and instead mediated an increased cytotoxicity that correlated with target density of CD58. The CD58 antibody-enhanced killing was dependent not only on FcRγIII but also on CD2 and ICAM-1/CD18. To further elucidate the mechanism of this CD58 antibody-dependent cellular cytotoxicity (ADCC), another antibody was tested. Trastuzumab (Herceptin), a humanized antibody against HER2/neu, mediated potent ADCC against all the HER2/neu positive breast cancer targets. Unlike CD58 antibody-mediated ADCC, Herceptin ADCC was minimally affected by blocking antibodies to CD2 or ICAM-1/CD18, which suggests a different mechanism of action. This study shows that multiple mechanisms are involved in NK cell lysis of breast cancer targets, that none of the targets are inherently resistant to killing, and that two distinct mechanisms of ADCC can target immunotherapy to breast cancer cells.

Keywords: Natural killer cell—Antibody-dependent cellular cytotoxicity—Breast cancer—Interleukin 2—Immunotherapy

Introduction

Breast cancer incidence continues to increase among western women, with a 12% cumulative lifetime risk of developing the disease [1]. Although great progress has been made in patients with low-stage disease and favorable tumor characteristics, surgery, radiation therapy, and chemotherapy are still inadequate for high-risk or recurrent breast cancer. Autologous stem cell transplantation has been used as a treatment for breast cancer, but success is limited by a high rate of disease recurrence. Less than 40% of patients with good risk features obtain long-term disease-free survival, which suggests that preparative regimens are unable to eradicate all clonogenic tumor [2]. Results in patients with poor prognosis disease (organ involvement or chemotherapy resistance) are even worse, and long-term disease-free survival is rarely seen [3,4]. Although donor lymphocyte infusions have shown promising graft-vs-tumor effects in patients who relapse after allogeneic bone marrow transplantation [5], the potential of immunotherapy in patients with breast cancer remains unknown.

Natural killer (NK) cells are a phenotypically distinct population of lymphocytes ($CD56^+/CD3^-$) that were first identified by their ability to lyse tumor cells without prior immunization. They mediate both major histocompatibility (MHC)-independent and antibody-dependent killing of tumors and virally infected cells. Additionally, they proliferate and secrete cytokines on activation. Interleukin 2 (IL-2) activation of NK cells induces proliferation and increases cytotoxicity against a wide range of targets not susceptible to lysis by resting NK cells [6]. Antibody-dependent cellular cytotoxicity (ADCC) by NK cells is mediated by binding of FcRγIII (CD16) to the Fc portion of antibodies, which initiates a sequence of cellular events culminating in the release of cytotoxic, granzyme-containing granules [7]. Different signaling pathways are engaged in the process of natural cytotoxicity by which NK cells lyse susceptible targets such as tumors or virally infected cells [8]. Although NK cell killing is non-MHC restricted in that it does not require class I MHC for target recognition. NK cells express reper-

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toires of immunoglobulin-like killer inhibitory receptors, which recognize class I and may influence the balance of whether target cell lysis occurs by engaging an overriding inhibitory signal [9]. Although NK cells do not have antigen-specific receptors, the receptor/ligand pairs CD2/LFA-3 and LFA-1/ICAM-1 are involved in NK cell/target interactions [10,11]. Whether or not a target is killed by NK cells is determined by a net balance of these positive and negative signals [12]. To improve current immunotherapy, we investigated the mechanisms of NK cell recognition and lysis of breast cancer targets.

Materials and methods

Study population

Peripheral blood or marrow was obtained from normal donors or from patients after informed consent using guidelines approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota. Peripheral blood mononuclear cells (PBMNC) or bone marrow mononuclear cells (BMMNC) were obtained by Ficoll-Hypaque (specific gravity 1.077) (Sigma Diagnostics, St. Louis, MO) density gradient centrifugation.

Normal NK populations

In initial studies to determine the effector specificity of breast cancer targets, PBMNC were sorted from the same donor for $CD4^+$ cells, $CD8^+$ cells, or $CD4^-/CD8^-$ cells. The CD4 and CD8 populations were cultured with irradiated mononuclear cells, 10 ng/mL OKT3 (Ortho Biotech, Raritan, NJ), and 1,000 U/mL IL-2 (a gift from Amgen, Thousand Oaks, CA) to yield greater than 98% pure populations of IL-2–activated $CD4^+$ or $CD8^+$ T cells. The $CD4^-/CD8^-$ population was cultured with irradiated mononuclear cells and 1,000 U/mL IL-2 to obtain an activated NK population devoid of T cells (<1%). In all subsequent studies, NK cells were enriched using a MACS column as specified by the manufacturer (Miltenyi, Auburn, CA). $CD56^+/CD3^-$ or $CD56^+/CD2^-$ NK cells were isolated by flow cytometry as described previously [6]. IL-2-activated NK cells were generated using PBMNC depleted of CD5/CD8 cells to enrich for NK cells and autologous monocytes as previously described [6] or by coculture of sorted $CD56^+/CD3^-$ NK cells on the murine stromal cell line, M210-B4 [13]. For both methods, NK cells were activated and expanded using an NK media supplemented with 1,000 U/mL IL-2 for 14 to 21 days prior to use. NK media consisted of a 2:1 (v/v) mix of DMEM/ Ham's F12-based medium (Gibco Laboratories, Grand Island, NY) supplemented with 24 μM 2-mercaptoethanol, 50 μM ethanolamine, 20 mg/L L-ascorbic acid, 5 μg/L sodium selenite (Na_2SeO_3), 100 U/mL penicillin, 100 U/mL streptomycin (Gibco), and 10% heat-inactivated human AB serum (North American Biologicals, Miami, FL) [14]. Resultant populations were greater than 90% $CD56^+/CD3^-$ cells.

NK populations from patients treated with subcutaneous IL-2

PBMNC were obtained from patients enrolled on a clinical trial of posttransplant immunotherapy with daily subcutaneous IL-2 (1.75 MU/m^2/day; Chiron Therapeutics, Emeryville, CA). The details of the clinical trial eligibility and safety of the phase I study have been described [15]. Briefly, patients were eligible for immunotherapy when they were beyond 30 days after transplant, engrafted, off growth factors, transfusion independent, outpatients, free of infections, and had good performance status. Patient samples, enriched in vivo for NK cells by IL-2 therapy, were used as fresh PBMNC without further purification.

Generation of $CD56^+/CD16^-$ NK cells

$CD34^+/Lin^-/CD38^-$ cells were isolated from normal BMMNC as described and plated in NK medium in direct contact with the murine fetal liver cell line, AFT024 [16]. Progenitors were plated (1,000 cells/well) in 96-well plates and supplemented with 1,000 U/mL IL-2, 10 ng/mL flt3 ligand (FL: a gift from Immunex, Seattle, WA), 20 ng/mL c-kit ligand (KL or stem cell factor, a gift from Amgen), 20 ng/mL interleukin-7 (IL-7; R&D Systems, Minneapolis, MN), and a one-time addition at culture initiation of 5 ng/mL IL-3 (R&D Systems). Cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2 and the medium was half changed weekly with the indicated cytokines (without IL-3). After 5 weeks, cultures were transferred to T-25 flasks and cultured for an additional 2 weeks with IL-2 alone.

Cell lines

The human breast cancer cell lines were obtained from Dr. David Kiang (University of Minnesota, Minneapolis, MN). MCF-7 was cultured in modified Eagle's medium (MEM; Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone Laboratories, Logan, UT), 0.2 μg/mL insulin, 50 U/mL penicillin, 50 μg/mL streptomycin, and 1% sodium pyruvate. T47-D cells were cultured in RPMI 1640 (Gibco), supplemented with 10% heat-inactivated FCS, 0.1 μg/mL insulin, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine. SKBR-3, BT-20, and MDA-MB-231 were cultured in MEM (Gibco), supplemented with 10% heat-inactivated FCS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine. In some experiments, cells were incubated with 1,000 U/mL interferon γ (Genzyme, Cambridge, MA). Cells were grown in monolayers in T-150 flasks (Corning, Cambridge, MA) at 37°C in a humidified atmosphere with 5% CO_2 prior to use.

Cytotoxicity, immunophenotyping, and antibodies

Cytotoxicity assays were performed at the indicated effector to target ratios (E:T) using resting or IL-2–activated NK cells against cell lines in a 4-hour ^{51}Cr release assay [17]. Monoclonal antibodies against CD58 (IgG2a, AICD58; Immunotech), CD58 (IgG2a, BRIC-5, Biosource), ICAM-1 (IgG1, 84H10, Immunotech), HER2/neu (IgG1, 2G11, Biosource), Trastuzumab (Herceptin, a humanized IgG1 antibody, Genentech, Inc., San Francisco, CA), and control IgG2a (X39, Becton Dickinson, San Jose , CA) were added to targets. CD18 (IgG3, P4H9, Gibco), CD2 (IgG2a, S5.2; Becton Dickinson), and CD16 (IgG1, 3G8, Immunotech) were added to the NK cells. All antibodies were added at a concentration of 10 μg/mL unless otherwise indicated 30 minutes prior to each assay and remained for the duration of the 4-hour incubation. Phenotype analyses were performed with a FACSCalibur (Becton Dickinson) and CELLQuest software (Becton Dickinson) using antibodies ICAM-1-PE (Becton Dickinson), CD58-PE (Immunotech), HER2/neu-FITC (Biosource), CD56-PE (Becton Dickinson), and CD16-FITC (Becton Dickinson).

Statistics

Results of experimental points obtained from multiple experiments were reported as mean ± 1 SEM. Significance levels were determined by two-sided Student's *t*-test.

Results

NK killing of breast cancer targets

The ability of NK cells to lyse breast cancer targets was assessed in vitro. Because IL-2 alone cannot efficiently expand human NK cells in vitro, coculture with autologous monocytes or stromal feeders was used. The NK expansion after 14 to 21 days using either of these two methods (NK cells cocultured with monocytes [6] or NK cells cocultured on the M210-B4 cell line [13]) is between 50- and 200-fold. These expanded populations have greater NK cell purity (>90% $CD56^+/CD3^-$) than traditional lymphokine-activated killer cells, which contain a heterogeneous mixture of NK cells and T cells. Resting purified $CD56^+/CD3^-$ NK cells from normal donors (E:T 6.6:1) exhibited low lytic activity against all of the breast cancer targets (less than 10% specific lysis, n = 4, data not shown). In contrast, activation and expansion of NK cells with 1,000 U/mL IL-2 and accessory cells resulted in an increase in cytotoxicity against all breast cancer targets. The cytotoxicity was mediated solely by the $CD56^+/CD3^-$-activated NK cells, and bulk IL-2–activated $CD4^+$ or $CD8^+$ T cells did not contribute to target lysis [data shown for MCF-7 (Fig. 1A)]. These activated NK populations exhibited significant but variable cytotoxicity against five breast cancer cell lines (MCF-7, T47D, MDA-MB-231, BT-20, SKBR-3). All of these cell lines, with the exception of BT-20, were originally derived from pleural effusions of patients with metastatic breast cancer [18]. The MCF-7, T47D and MDA-MB-231 targets were consistently more sensitive to activated NK cell lysis compared to the BT-20 or SKBR-3 targets, which were killed less efficiently (Fig. 1B).

Role of β2 integrin/ICAM interactions in NK cell killing of breast cancer targets

β2 Integrin (CD18) recognition of ICAM-1 is a described mechanism of recognition for NK-mediated killing of various fresh and cultured tumor targets [18,19]. To test the role of this recognition mechanism against breast cancer targets, several experiments were performed. Breast cancer targets were evaluated for ICAM-1 expression by flow cytometry after culture in their respective media with or without the addition of interferon-γ, a known inducer of ICAM-1 expression [20]. SKBR-3, a cell line killed less efficiently by activated NK cells and with low basal expression of surface ICAM-1, significantly increased ICAM-1 expression after a 24-hour preincubation of targets with interferon γ (from a mean channel fluorescence [MCF] of 440 to 818). SKBR-3 targets then were tested in cytotoxicity assays to determine whether the increased ICAM-1 expression increased their susceptibility to activated NK cell lysis. In contrast to our hypothesis, interferon γ treatment of targets made them more resistant to lysis despite the increase in ICAM-1 surface expression (data not shown), which suggests that other factors play a role in determining target sensitivity.

The contribution of β2 integrin/ICAM-1 interactions toward the lysis of breast cancer targets was assessed directly in experiments with blocking antibodies against ICAM-1, CD18, or the combination. Consistent with data shown in Figure 1B, the baseline lysis of MCF-7 targets was highest and lysis was not significantly inhibited with any of the single antibodies or combinations tested (data not shown). In contrast, blocking antibodies alone or in combination variably inhibited lysis of the remaining breast cancer targets (Fig. 2). The combination of antibodies resulted in greater inhibition than single antibodies, except for SKBR-3 where CD18 blocking and the combination of CD18 and ICAM-1 resulted in similar inhibition. There was no significant difference in target lysis inhibition with ICAM-1 blocking for the breast cancer targets with the highest surface ICAM-1 expression (MDA-MB-231 [MCF = 891], BT-20 [MCF = 799]) and the targets with the lowest expression (T47D [MCF = 264], SKBR-3 [MCF = 440]). Furthermore, sur-

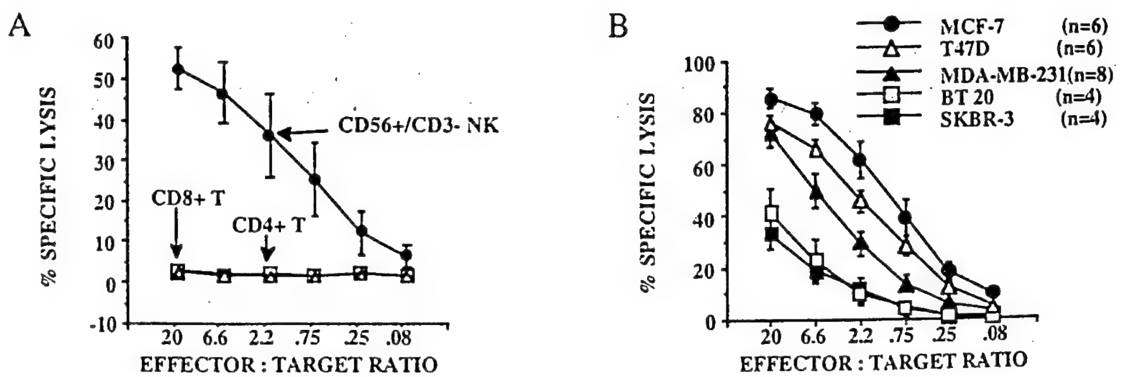

Figure 1. IL-2–activated NK cells mediate significant cytotoxicity against breast cancer targets in vitro. (A) IL-2–activated populations of $CD4^+$, $CD8^+$, and NK cells were all generated from blood of normal donors and tested in cytotoxicity assays against the MCF-7 target. Activated NK cells (92 ± 2% $CD56^+/CD3^-$), but not $CD4^+$ or $CD8^+$ T cells, exhibited lysis of MCF-7 targets (n = 3). (B) NK cells and autologous monocytes were obtained from normal donors and activated for 14 to 18 days with 1,000 U/mL IL-2. The resultant populations (90 ± 3% $CD56^+/CD3^-$) were tested in cytotoxicity assays against five breast cancer cell lines (MCF-7, T47D, MDA-MB-231, BT-20, SKBR-3).

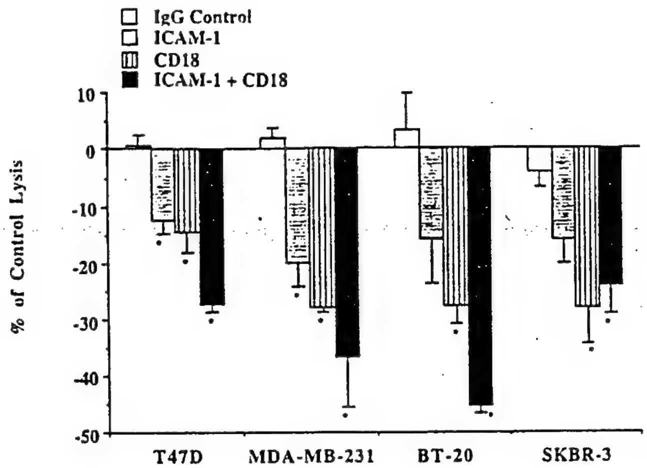

Figure 2. ICAM-1/CD18 interactions are involved in IL-2–activated NK lysis of breast cancer targets. IL-2–activated NK cells (96 ± 2% CD56⁺/CD3⁻) from normal donors were tested in cytotoxicity assays against breast cancer targets at an effector to target ratio of 4:1. Specific lysis was calculated for each target in the absence of antibody for T47D (79 ± 3.4%), MDA-MB-231 (70 ± 2.7%), BT-20 (45 ± 9.3%), and SKBR-3 (53 ± 9%). Data are presented as the percent of control with each antibody or combination as follows: [(% lysis with antibody − % lysis without antibody)/(% lysis without antibody)]. Each bar represents mean ± SEM of 4 to 6 individual experiments analyzed in duplicate. There was significant inhibition of specific lysis for each breast cancer target involving recognition through β2 integrins on NK cells. *$p < 0.05$.

face expression of ICAM-1 did not correlate with sensitivity to killing.

Role of CD2/LFA-3 interactions in NK cell killing of breast cancer targets

In addition to β2 integrin recognition of targets, the interaction of CD2 on NK cells with CD58 on some targets has been described [11,21]. To test the role of this ligand pair, experiments were performed using antibodies in cytotoxicity assays to determine their effect on breast cancer target cell lysis. Addition of anti-CD2 antibodies to NK cells resulted in less than 10% change in specific lysis, which was not significantly different from controls without antibody or with mouse IgG. In contrast, addition of the CD58 antibody (AICD58) to targets consistently increased killing of MDA-MB-231, BT-20 and SKBR-3 (data not shown). Addition of the CD58 antibody (AICD58) alone to targets without effectors did not result in lysis, which suggests that CD58 antibody may be functioning through ADCC.

Antibodies against CD58 mediate ADCC

Breast cancer cell lines were phenotyped for surface expression of CD58 and HER2/neu, a known antigen overexpressed on some breast cancers. All targets were positive for CD58 and HER2/neu with variable expression (Table 1). BT-20 expressed the highest surface density of CD58, whereas T47D was the least positive. This correlated well with the increased CD58 antibody enhanced killing of BT-20 by IL-2–activated NK cells. HER2/neu expression was highest on SKBR-3 and lowest on MDA-MD-231. There was no apparent correlation between the relative expression of CD58 and HER2/neu expression between the breast cancer targets tested.

Resting CD56⁺/CD3⁻ NK cells from normal donors were purified using flow cytometry and tested without further activation in cytotoxicity assays against all five breast cancer targets. In addition to the CD58 antibody that increased lysis with IL-2–activated NK cells, another CD58 clone (BRIC-5, IgG2a) was tested. As expected, the baseline killing of breast cancer targets by NK cells without IL-2 activation was low at E:T 10:1. There was no difference in

Table 1. Mean channel fluorescence of CD58 and HER2/neu on breast cancer cell lines

Cell line	CD58(LFA-3)	HER2/neu
MCF-7	153	62
T47D	57	83
MDA-MB-231	147	54
BT-20	286	105
SKBR-3	90	1,676

Mean channel fluorescence (MCF) of the isotype control was between 6 and 22 for all samples.

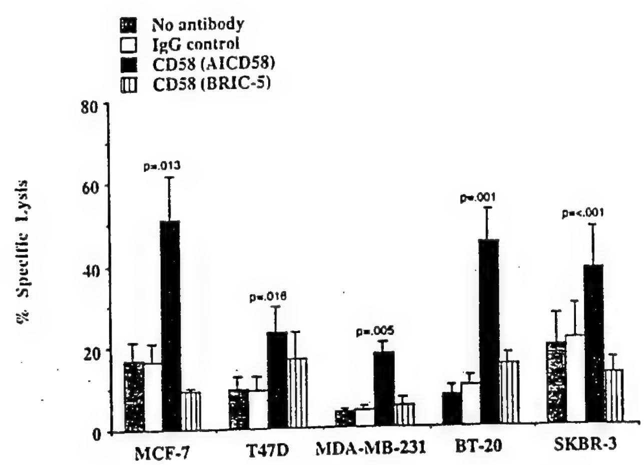

Figure 3. Incubation of breast cancer targets with CD58 (AICD58) antibody enhances antibody-dependent cellular cytotoxicity. Resting NK cells were purified from normal donors by flow cytometry (>97% CD56⁺/CD3⁻) and incubated with breast cancer targets at an effector to target ratio of 10:1 without activation with IL-2. Cytotoxicity was performed without (n = 6) or with the addition of mouse IgG (n = 6), anti-CD58 (clone AICD58, IgG2a, n = 6), or anti-CD58 (clone BRIC-5, IgG2a, n = 2). Each bar represents the percent specific lysis (mean ± SEM) of experiments analyzed in duplicate. The CD58 (AICD58) antibody significantly enhanced lysis of all breast cancer targets as indicated compared to the mouse IgG control. No other differences were found.

lysis between assays performed without antibody as compared to those with IgG control or with the CD58 (BRIC-5) antibody. In contrast, the CD58 (AICD58) antibody significantly increased killing against all breast cancer targets tested (Fig. 3). The fold increase in the mean specific lysis for the cell lines with the highest CD58 expression (MCF-7, MDA-MB-231, BT-20) was greater than the fold increase in mean specific lysis for the breast cancer cell lines with the lowest CD58 expression (T47D, SKBR-3). Titration experiments with the CD58 (AICD58) antibody and the BT-20 target showed enhanced lysis down to an antibody concentration of 0.1 μg/mL (n = 2).

In vivo activated mononuclear cells collected from patients treated with subcutaneous IL-2 exhibited relatively low cytotoxicity against all the breast cancer cell lines. MCF-7, which is consistently the most susceptible to lysis by activated NK cells, is not lysed by patient mononuclear cells collected prior to starting IL-2 therapy (2.4 ± 1.1% lysis at E:T 60:1). Twenty-eight days of subcutaneous daily IL-2 treatment induced a modest increase in cytotoxicity by patient mononuclear cells against MCF-7 (19 ± 3.1% lysis at E:T 60:1) and less of an increase against other cell lines (data not shown). To measure the effect of the CD58 (AICD58) antibody on NK cells expanded in vivo, we tested mononuclear cells from patients receiving subcutaneous IL-2 against BT-20, the breast cancer target with the highest expression of CD58. Specific lysis of BT-20 targets was approximately 10% when tested without antibody or with an isotype-matched IgG2a control antibody. Similar to the results obtained with normal donor cells, addition of the CD58 (AICD58) antibody increased target lysis to 60% (Fig. 4, left), six times greater than control.

To assess whether the CD58 (AICD58) antibody-enhanced killing was dependent on Fc receptors, populations of CD16 (FcRγIII) negative NK cells were generated from IL-2–dependent, stromal-dependent long-term culture using marrow-derived CD34⁺ progenitor cells. CD56⁻/CD3⁻ NK cells were generated from CD34⁺/Lin⁻/CD38⁻ cells that coexpressed 1.1 ± 0.3% CD16 (n = 4). In contrast to the normal donor NK cells and the in vivo activated patient mononuclear cells used in the previous assays, the IL-2–activated NK progeny (CD56⁺/CD16⁻) derived from marrow progenitors did not mediate ADCC (Fig. 4, right).

Blocking antibodies were used to determine which accessory molecules were necessary for the enhanced killing by the CD58 (AICD58) antibody. Both mononuclear cells from patients receiving IL-2 (data not shown) and normal donor purified NK cells (n = 4) were tested against the breast cancer target BT-20. Cytotoxicity assays were performed with CD58 antibody alone and in combination with ICAM-1, CD18, CD2, and CD16. Blocking ICAM-1/CD18 interactions, CD2 alone or CD16 alone significantly inhibited the enhanced killing by the CD58 (AICD58) antibody, and the combinations completely abrogated the enhanced effect.

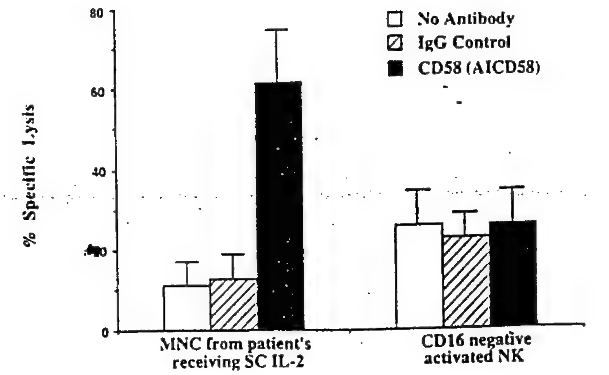

Figure 4. CD58-mediated antibody-dependent cellular cytotoxicity (ADCC) increases lytic activity in mononuclear cells (MNC) from patients receiving subcutaneous (SC) IL-2 but not in NK cells, which are CD16 negative. Peripheral blood mononuclear cells were obtained from patients treated for 14 to 28 days with SC IL-2 (1.75×10^6 U/m²/day) on a protocol to prevent relapse after autologous stem cell transplantation. Patient mononuclear cells (63 ± 8% CD56⁺/CD3⁻, of which 87 ± 2% expressed FcRγIII [CD16]), without further IL-2 activation, were tested against the BT-20 breast cancer target at an effector to target ratio of 20:1. Cytotoxicity was significantly enhanced ($p < 0.05$, n = 4 patient samples performed in duplicate) when targets were pretreated with CD58 (AICD58) antibody compared to no antibody or IgG control. The requirement for FcRγIII (CD16) on NK cells was tested further by generating NK cells from marrow CD34⁺ progenitors in a long-term NK cell differentiation culture. IL-2 cultured NK cell progeny (97 ± 1% CD56⁺/CD3⁻, of which 1.1 ± 0.2% expressed FcRγIII [CD16]) were tested against BT-20 at an effector to target ratio of 10:1. Unlike normal donor or patient-derived NK cells, the CD16⁻ NK cells derived from marrow progenitors were unable to mediate ADCC when targets were pretreated with the CD58 (AICD58) antibody (n = 4).

Trastuzumab (Herceptin) mediates ADCC through a different mechanism

If the CD58 antibody was mediating classic ADCC by signaling through FcRγIII, the significant blocking effect of CD2 would remain unexplained. To further explore this finding, we tested another antibody that mediates ADCC. Herceptin is a humanized antibody against HER2/neu engineered by inserting the complementary determining regions of a murine antibody (clone 4D5) into the framework of a consensus human IgG1 [22]. In contrast to the HER2/neu murine antibody (clone 2G11, IgG1), which did not mediate ADCC (n = 2, data not shown), Herceptin added to targets and normal CD56⁺/CD3⁻ NK cells significantly enhanced killing of all breast cancer targets except for MDA-MB-231, the target with the lowest HER2/neu expression (Fig. 5). Titration experiments with the Herceptin antibody and the SKBR-3 target, the target with the highest expression of HER2/neu, showed enhanced lysis down to an antibody concentration of 0.01 μg/mL (n = 2), which was the concentration used in subsequent ADCC blocking experiments. Marrow-derived CD16⁻ NK cells did not augment killing of SKBR-3 targets in the presence of Herceptin (n = 6, data not shown). Similar to CD58 (AICD58) ADCC, Herceptin augmented killing by resting blood NK cells was also FcRγIII (CD16) dependent

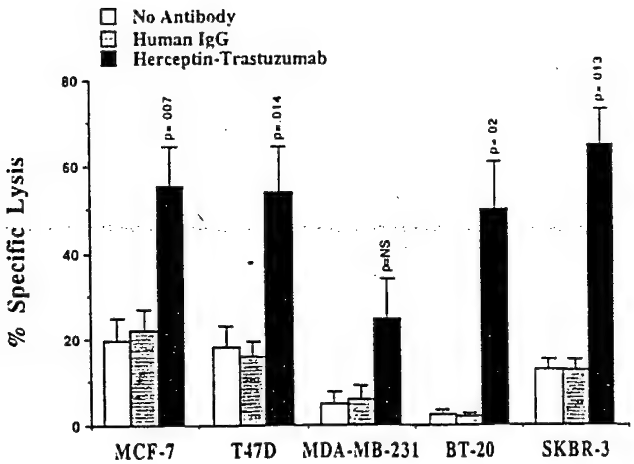

Figure 5. Incubation of breast cancer targets with Herceptin (humanized anti-HER2/neu) mediates antibody-dependent cellular cytotoxicity. Resting NK cells were purified from normal donors and incubated with breast cancer targets at an effector to target ratio of 10:1 without activation with IL-2. Cytotoxicity was performed without antibody or with the addition of human IgG or Herceptin at 10 μg/mL (n = 4 in triplicate). The Herceptin antibody significantly enhanced lysis of all breast cancer targets except MDA-MB-231, as indicated. Cytotoxicity with Herceptin is compared to the human IgG control.

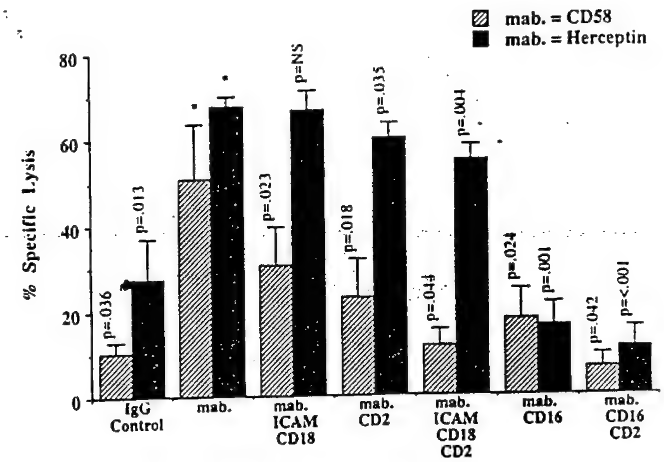

Figure 6. CD58 antibody-mediated antibody-dependent cellular cytotoxicity (ADCC) but not Herceptin-mediated ADCC is decreased by blocking ICAM-1/CD18 and CD2. Sorted (>97% CD56$^+$/CD3$^-$) normal donor NK cells (effector to target ratio 10:1), without further IL-2 activation, were tested against the BT-20 (hatched bars) or SKBR-3 (black bars) breast cancer targets along with antibodies that mediate ADCC, CD58 (hatched bars) and Herceptin (0.01 μg/mL, black bars), respectively. Cytotoxicity was performed in the presence or absence of blocking antibodies as indicated (n = 4 donors in triplicate). Both the CD58 (AICD58) antibody and Herceptin significantly increased lytic activity compared to IgG control. In the presence of CD58 antibody, addition of antibodies to block ICAM-1, CD18, and CD2 resulted in suppression of CD58 antibody-mediated ADCC, whereas Herceptin ADCC decreased only slightly. All *p* values listed are compared to the addition of monoclonal antibody (mab) alone (*).

as shown using blocking antibodies (Fig. 6). In contrast to CD58 (AICD58) ADCC, which was decreased by nearly 50% by CD2 or ICAM-1/CD18, these same blocking antibodies had less of an effect on Herceptin ADCC (Fig. 6). Whereas blocking both CD2 and ICAM-1/CD18 completely abrogated CD58 (AICD58) ADCC, ADCC with Herceptin was only slightly blocked with the same combination of antibodies.

Although both antibodies [CD58 (AICD58) and Herceptin] result in CD16-dependent killing, blocking experiments suggest different interactions with accessory receptor/ligand pairs. CD58 (AICD58)-mediated ADCC appears to be CD2 dependent, whereas Herceptin ADCC is minimally affected by blocking CD2. To further test this, we used a subset of NK cells that is CD56 and CD16 positive but CD2 negative. This subset, which generally comprises 10 to 40% of normal blood NK cells [23], was purified by flow cytometry (Fig. 7A). Secondary staining of CD56$^+$/CD2$^-$ sorted NK cells showed that greater than 80% expressed CD16. CD56$^+$/CD16$^+$/CD2$^-$ NK cells were still able to augment target lysis of Herceptin-treated SKBR-3 targets, which suggests a CD2-independent mechanism of ADCC signaling through CD16. In contrast, CD56$^+$/CD16$^-$/CD2$^-$ NK cells did not lyse CD58 (AICD58) antibody-treated BT-20 targets, which confirms the CD2 dependence of this ADCC and the lack of triggering through CD16 alone (Fig. 7B).

Discussion

Breast cancer relapse remains a major clinical problem even after dose-intensive therapy such as autologous transplantation. We hypothesize that immunotherapy used in a minimal residual disease setting after transplantation may serve as a noncross-resistant therapy to prevent relapse. Although NK cells are among the first immune effectors to reconstitute after stem cell transplantation, resting NK cells do not exhibit activity against breast cancer targets until they are activated with exogenous IL-2. We are concerned that well-tolerated doses of IL-2 alone may not be efficacious.

Normal NK cells were found to exhibit variable killing of five breast cancer cell lines, whereas bulk CD4$^+$ or CD8$^+$ T cells exhibited no activity. SKBR-3, the target with the highest HER2/neu expression, was the most resistant to IL-2–activated NK lysis, as has been observed by others [24]. The role of ICAM-1 in effector recognition of targets has been studied extensively, and it seemed reasonable to hypothesize that sensitivity to lysis may correlate with the relative expression of ICAM-1 on targets. This notion was supported by data from Budinsky et al. [19], who found that primary breast cancer cells expressed lower ICAM-1 than benign breast tissue, suggesting that tumors may escape immune recognition by decreasing their ICAM-1 expression. However, our results and those of others do not support this premise. There was no correlation between surface expression of ICAM-1 and target sensitivity to NK cell lysis, and induction of ICAM-1 on targets failed to make them more

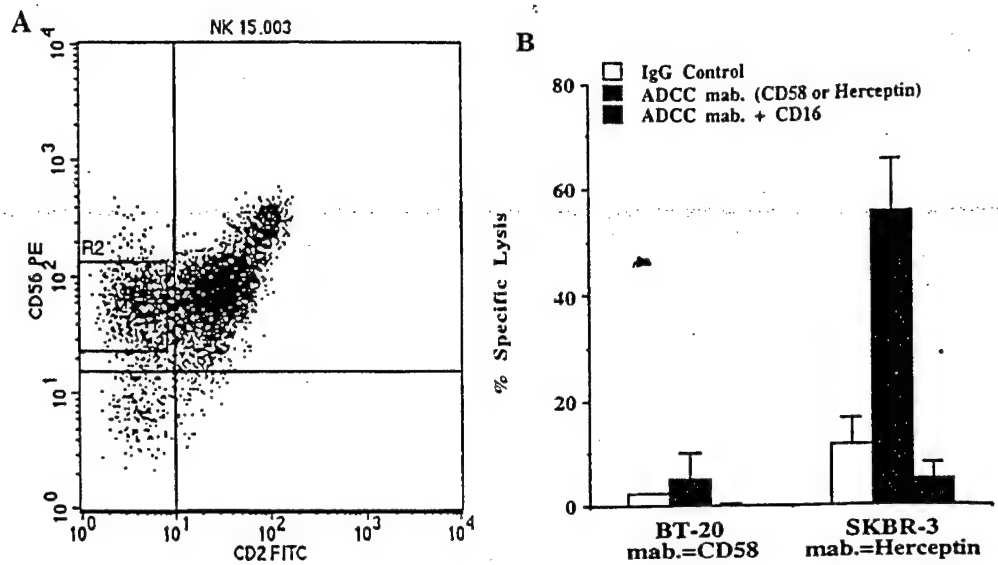

Figure 7. CD58-mediated antibody-dependent cellular cytotoxicity (ADCC) but not Herceptin-mediated ADCC is dependent on CD2. **(A)** CD56$^+$/CD2$^-$ NK cells were sorted by flow cytometry. An example of a representative sort with the collection window designated R2 is shown. **(B)** CD56$^+$/CD2$^-$ NK cells, which are predominantly CD16 positive, were tested in cytotoxicity assays using the breast cancer target and antibodies as indicated. The CD2- NK cells exhibited CD16-dependent killing of Herceptin-treated SKBR-3 targets but had no effect on CD58 (AICD58) antibody-treated BT-20 targets.

susceptible. These results do not exclude ICAM-1 as playing an important role in NK killing. They merely suggest that ICAM-1 interactions are among the many factors determining target cell lysis. In agreement with our data, Gwin et al. [21] showed that increased ICAM-1 expression by interferon on A-375 (melanoma) and Daudi (lymphoma) tumor cells increased effector/target conjugation but decreased killing. ICAM-1 may be more important for initial recognition, whereas other interactions and postbinding events further modulate the fate of whether a target is killed [20]. The concurrent upregulation of class I MHC, also induced by interferon γ, may explain this observation. This is of particular interest in light of the multiple structures and variants of class I recognizing receptors found on all NK cells, although their physiologic relevance in cancer is still uncertain.

The combination of ICAM-1 and CD18-blocking antibodies did not result in greater than 50% inhibition for any of the targets, which suggests that other mechanisms were operant. This led to experiments exploring the role of CD2/CD58 in the lytic mechanism. Although interrupting the cell/target interaction with antibody to either CD2 [25] or CD58 [11,21] has been shown to inhibit target cell lysis, the CD58 (AICD58) antibody used here mediated the opposite effect and enhanced target killing. Consistent with ADCC, the CD58 (AICD58) antibody effects were independent of IL-2 activation and NK cell CD16 (FcRγIII) was required in the process. We used unique differences between mature NK cells and those derived from long-term cultures of marrow progenitors to generate NK cells that were CD16 negative. We have shown that these cells exhibit characteristic lysis of K562 targets demonstrating that their lytic machinery is intact [26,27]. The failure of the CD58 (AICD58) antibody to enhance killing by the marrow progenitor-derived NK cells demonstrates a requirement for CD16, which is consistent with Fc-mediated ADCC. The ability to generate functional NK cells lacking specific receptors can be a useful tool to dissect the complex interactions involved in NK cell killing.

ADCC by NK cells is mediated through binding of IgG immune complexes or antibody-coated targets to the low-affinity Fc receptor for IgG, FcRγIII. The α subunit of CD16, which binds the Fc portion of IgG molecules, associates noncovalently with the signal-transducing molecules CD3ζ and FcεRI-γ [7]. It is thought that antigen density and structure, as well as the isotype specificity of Fc binding, all contribute to the induction of ADCC [28]. Human NK cells have been shown to exhibit ADCC using murine antibodies of several isotypes (IgG1, IgG2a, IgG2b, IgG3) [29, 30]. Others report a variation among individual donors in the NK response to IgG of different isotypes [31]. We found clone-specific anti-CD58–mediated ADCC. As both CD58 antibodies were isotype IgG2a, the inability of clone BRIC-5 to mediate ADCC may be due to epitope specificity or to some characteristic of tertiary structure. Similarly, we were not able to induce ADCC with anti-HER2/neu antibody clone 2G11 (IgG1), whereas several others have described ADCC using different clones of the same isotype [22,32].

Accessory cell molecules may play an important role in CD58 (AICD58) antibody-mediated ADCC. In addition to the primary role for CD16, our data also show a role for ICAM-1/LFA-1 interaction in ADCC similar to that de-

scribed by Lanier et al. [9]. We could not find any reports of ADCC mediated through CD58. However, antibodies against its ligand, CD2, have been shown to activate NK lysis when cross-linked to Fc receptor positive targets, a process called reverse ADCC or antibody-redirected lysis [9]. The finding that antibody against CD2 blocks CD58 (AICD58) antibody-mediated ADCC suggests that CD16 and CD2 may be colocalized on the NK cell surface. The CD58 antibody, linked to the NK cell Fc receptor (CD16), may serve as an anchor to increase the affinity of CD2 to its natural ligand. Blocking CD2 may sterically hinder this association, which suggests that the signal for target lysis may be through CD2 rather than CD16, as described for classic ADCC. Direct evidence to support this notion was obtained from experiments using a normal NK cell subset that is $CD56^{+}/CD16^{+}/CD2^{-}$. These $CD2^{-}$ NK cells were unable to enhance target lysis with the CD58 (AICD58) antibody, proving that CD16 alone is insufficient to mediate this mechanism of ADCC. This is contrasted to ADCC mediated by Herceptin, which exhibits classic ADCC through CD16, which is CD2 independent. Both antibodies still mediate ADCC when titrated down to low concentrations and recognize targets with a broad range of either LFA-3 or HER2/neu surface densities.

We have been studying whether IL-2–based immunotherapy has an anti-tumor effect that can be used as additional adjuvant therapy after stem cell transplantation to increase survival in patients with breast cancer. We have previously shown that subcutaneous IL-2 can be given safely to autologous transplant patients and that daily IL-2 in vivo expands NK cells that exhibit increased cytotoxicity against breast cancer targets [15]. Despite these promising results, the in vivo activity induced by subcutaneous IL-2 therapy after transplantation is submaximal when compared to NK cells activated ex vivo with a higher concentration of IL-2, which raises the possibility that current therapy may be insufficient to mediate a therapeutic response. We show that ADCC with the CD58 (AICD58) or Herceptin antibodies markedly enhances killing of breast cancer targets by two different mechanisms: one through CD16 signaling and one by increasing the affinity of a receptor (CD2) to its natural ligand (CD58). Our data suggest that monoclonal antibodies combined with subcutaneous IL-2, which expands NK cells 10-fold in vivo without loss of Fc function as shown here, may be a feasible method to increase the efficacy of immunotherapy against breast cancer. This strategy and others that target immunotherapy will be the focus of future investigations.

Acknowledgments

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References

1. Parker SL, Tong TT, Bolden S, Wingo PA (1997) Cancer statistics, 1997. CA Cancer J Clin 47:13
2. Antman KH, Rowlings PA, Vaughan WP, Pelz CJ, Fay JW, Fields KK, Freytes CO, Gale RP, Hillner BE, Holland HK, Kennedy MJ, Klein JP, et al (1997) High-dose chemotherapy with autologous hematopoietic stem-cell support for breast cancer in North America. J Clin Oncol 15:1870
3. Ayash LJ, Wheeler C, Fairclough D, Schwartz G, Reich E, Warren D, Schnipper L, Antman K, Frei EI, Elias A (1995) Prognostic factors for prolonged progression-free survival with high-dose chemotherapy with autologous stem-cell support for advanced breast cancer. J Clin Oncol 13:2043
4. Dunphy FR, Spitzer G, Fornoff JE, Yau JC, Huan SD, Dicke KA, Buzdar AU, Hortobagyi GN (1994) Factors predicting long-term survival for metastatic breast cancer patients treated with high-dose chemotherapy and bone marrow support [published erratum appears in Cancer 1994;74:773]. Cancer 73:2157
5. Or R, Ackerstein A, Nagler A, Kapelushnik J, Naparstek E, Samuel S, Amar A, Brautbar C, Slavin S (1998) Allogenic cell-mediated immunotherapy for breast cancer after autologous stem cell transplantation: a clinical pilot study. Cytokines Cell Mol Ther 4:1
6. Miller JS, Oelkers S, Verfaillie C, McGlave PB (1992) Role of monocytes in the expansion of human activated natural killer cells. Blood 80:2221
7. Leibson PJ (1997) Signal transduction during natural killer cell activation: inside the mind of a killer. Immunity 6:655
8. Leibson PJ (1995) Viewpoint: signal transduction during natural killer cell activation. Nat Immun 14:117
9. Lanier LL, Corliss B, Phillips JH (1997) Arousal and inhibition of human NK cells. Immunol Rev 155:145
10. Cervantes F, Pierson BA, McGlave PB, Verfaillie CM, Miller JS (1996) Autologous activated natural killer cells suppress primitive chronic myelogenous leukemia progenitors in long-term culture. Blood 87:2476
11. Altomonte M, Gloghini A, Bertola G, Gasparollo A, Carbone A, Ferrone S, Maio M (1993) Differential expression of cell adhesion molecules CD54/CD11a and CD58/CD2 by human melanoma cells and functional role in their interaction with cytotoxic cells. Cancer Res 53:3343
12. Raulet DH, Held W (1995) Natural killer cell receptors: the offs and ons of NK cell recognition. Cell 82:697
13. Pierson BA, Gupta K, Hu WS, Miller JS (1996) Human natural killer cell expansion is regulated by thrombospondin-mediated activation of TGF-b1 and independent accessory cell-derived contact and soluble factors. Blood 87:180
14. Pierson BA, McGlave PB, Hu WS, Miller JS (1995) Natural killer cell proliferation is dependent on human serum and is markedly increased utilizing an enriched supplemented basal medium. J Hematother 4:149
15. Miller JS, Tessmer-Tuck J, Pierson BA, Weisdorf D, McGlave P, Blazar BR, Katsanis E, Verfaillie C, Lebkowski J, Radford J, Burns LJ (1997) Low dose subcutaneous interleukin-2 after autologous transplantation generates sustained in vivo natural killer cell activity. Biol Blood Marrow Transplant 3:34

16. Miller JS, McCullar V, Punzel M, Lemischka IR, Moore KA (1999) Single adult human CD34+/Lin−/CD38− progenitors give rise to NK cells, B-lineage cells, dendritic cells and myeloid cells. Blood 93:96
17. Miller JS, Klingsporn S, Lund J, Perry EH, Verfaillie C, McGlave P (1994) Large-scale ex vivo expansion and activation of human natural killer cells for autologous therapy. Bone Marrow Transplant 14:555
18. Hart MK, Kornbluth J, Main EK, Spear BT, Taylor J, Wilson DB (1987) Lymphocyte function-associated antigen 1 (LFA-1) and natural killer (NK) cell activity: LFA-1 is not necessary for all killer:target cell interactions. Cell Immunol 109:306
19. Budinsky AC, Brodowicz T, Wiltschke C, Czerwenka K, Michl I, Krainer M, Zielinski CC (1997) Decreased expression of ICAM-1 and its induction by tumor necrosis factor on breast cancer cells in vitro. Int J Cancer 71:1086
20. Fady C, Gardner A, Gera JF, Lichtenstein A (1993) Interferon-gamma-induced increased sensitivity of HER2/neu-overexpressing tumor cells to lymphokine-activated killer cell lysis: importance of ICAM-1 in binding and post-binding events. Cancer Immunol Immunother 37:329
21. Gwin JL, Gercel-Taylor C, Taylor DD, Eisenberg B (1996) Role of LFA-3, ICAM-1 and MHC Class I on the sensitivity of human tumor cells to LAK cells. J Surg Res 60:129
22. Lewis GD, Figari I, Fendly B, Wong WL, Carter P, Gorman C, Shephard HM (1993) Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. Cancer Immunol Immunother 37:255
23. Pierson BA, Miller JS, Verfaillie C, McGlave PB, Hu WS (1994) Population dynamics of human activated natural killer cells in culture. Bioeng Biotechnol 43:685
24. Lichtenstein A, Berenson J, Gera JF, Waldburger K, Martinez-Maza O, Berek JS (1990) Resistance of human ovarian cancer cells to tumor necrosis factor and lymphokine-activated killer cells: correlation with expression of HER2/neu oncogenes. Cancer Res 50:7364
25. Zarcone D, Viale O, Cerruti G, Tenca C, Malorni W, Arancia G, Iosi F, Galandrini R, Velardi A, Moretta A, Grossi CE (1992) Antibodies to adhesion molecules inhibit the lytic function of MHC-unrestricted cytotoxic cells by preventing their activation. Cell Immunol 143:389
26. Miller JS, Verfaillie C, McGlave PB (1992) The generation of human natural killer cells from CD34+/DR− primitive progenitors in long-term bone marrow culture. Blood 80:2182
27. Miller JS, Alley KA, McGlave PB (1994) Differentiation of natural killer cells from human primitive marrow progenitors in a stroma based long term culture system: identification of a CD34+/CD7+ NK progenitor. Blood 83:2594
28. Hooijberg E, Sein JJ, Van Den Berk PCM, Hekman A (1996) Characterization of a series of isotype switch variants of a new CD20 monoclonal antibody. Hybridoma 15:23
29. Kipps TJ, Parham P, Punt J, Herzenberg LA (1985) Importance of immunoglobulin isotype in human antibody-dependent, cell-mediated cytotoxicity directed by murine monoclonal antibodies. J Exp Med 161:1
30. Ortaldo JR, Woodhouse C, Morgan AC, Herberman RB, Cheresh DA, Reisfeld R (1987) Analysis of effector cells in human antibody-dependent cellular cytotoxicity with murine monoclonal antibodies. J Immunol 138:3566
31. Galatiuc C, Gherman M, Metes D, Sulica A, DeLeo A, Whiteside TL, Herberman RB (1995) Natural killer (NK) activity in human rsponders and nonresponders to stimulation by anti-CD16 antibodies. Cell Immunol 163:167
32. Brodowicz T, Wiltschke C, Budinsky AC, Krainer M, Steger GG, Zielinske CC (1997) Soluable HER-2/neu neutralizes biologic effects of anti-HER-2/neu antibody on breast cancer cells in vitro. Int J Cancer 73:875

EXPERIMENTAL HEMATOLOGY

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Enhancement of the anti-tumor activity of a peripheral blood progenitor cell graft by mobilization with interleukin 2 plus granulocyte colony-stimulating factor in patients with advanced breast cancer

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Objective. Autologous interleukin 2 (IL-2)-activated natural killer (NK) cells kill a broad spectrum of tumor targets, including breast cancer. We hypothesized that mobilization with IL-2 and granulocyte colony-stimulating factor (G-CSF) for collection of peripheral blood progenitor cells (PBPC) may enhance the anti-tumor activity of the graft in autograft recipients. We determined the dose-limiting toxicity and maximum tolerated dose of subcutaneous IL-2 given with G-CSF for PBPC mobilization, the ability of IL-2 + G-CSF mobilized stem cells to reconstitute hematopoiesis, and the in vitro immunologic function of the graft in patients with advanced breast cancer.

Materials and Methods. Forty-three women with stage IIIA/B or metastatic breast cancer underwent mobilization of PBPC with IL-2 administered subcutaneously for 14 days along with G-CSF for the latter 7 days. IL-2 was given in a dose-escalated manner, with the maximum tolerated dose determined to be 1.75×10^6 IU/m^2/day. Fifteen women with stage IIIA/B or metastatic breast cancer underwent G-CSF mobilization alone and served as a control group. Fifty-two percent of the patients mobilized with IL-2 at the maximum tolerated dose reached the target number of CD34$^+$ cells for transplantation with three aphereses compared to 93% of control patients who were mobilized with G-CSF alone.

Results. There was no significant impact on time to engraftment of neutrophils or platelets using either mobilization regimen. The addition of subcutaneous IL-2 to mobilization increased the cytotoxicity of IL-2–activated mononuclear cells from the PBPC product against the breast cancer cell target, MCF-7, and increased the percentage of NK cells and activated T cells in the PBPC product. The enhanced NK cell number was sustained in the early posttransplant period. IL-2 + G-CSF mobilization is safe, may lead to a more immunologically functional graft without impairing hematologic recovery, and thus merits further exploration to evaluate the clinical anti-tumor efficacy of these immunocompetent grafts.

Conclusions. Limitations of this combined approach to stem cell mobilization include a decrease in the number of CD34$^+$ cells mobilized with the combined cytokines and the short duration of the increased number of anti-tumor effector cells after transplant.

Keywords: Breast cancer—Interleukin 2—Granulocyte colony-stimulating factor—Mobilization—Peripheral blood progenitor cells

Introduction

Breast cancer is the most common malignant neoplasm in women and is the second leading cause of cancer-related death in women in the United States. High-dose chemotherapy with autologous peripheral stem cell rescue permits high-dose intensive chemotherapy treatment and may be beneficial for certain patients with advanced breast cancer [1]. However, a high rate of relapse accounts for the majority of patient deaths following transplantation [2,3]. Patients typically relapse at sites of prior disease, suggesting that minimal residual disease may not have been eradicated by

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the preparative regimen. Attempts to improve on this relapse rate have included incorporation of sequential cycles of high-dose chemotherapy [4,5], posttransplant chemotherapy [6,7], and immunotherapy [8,9].

Autologous interleukin 2 (IL-2)-activated natural killer (NK) cells kill a broad spectrum of tumor targets, including breast cancer. We have shown that administration of granulocyte colony-stimulating factor (G-CSF) to normal donors results in suppression of NK cell function in peripheral blood progenitor cell (PBPC) products [10]. Because NK cell function can be markedly augmented by IL-2, we hypothesized that in vivo mobilization with IL-2 and G-CSF for collection of PBPCs used for hematopoietic rescue may reverse the NK suppressive effects of G-CSF and lead to an in vivo enhanced graft-vs-tumor effect in the immediate posttransplant period. We report results of a phase I trial designed to determine the dose-limiting toxicity and maximum tolerated dose of subcutaneous IL-2 given with G-CSF for stem cell mobilization, the ability of IL-2 + G-CSF mobilized stem cells to reconstitute hematopoiesis, and the in vitro immunologic function of the graft.

Materials and methods

Patient characteristics and eligibility criteria

Forty-three consecutive women 18 to 65 years of age with chemosensitive stage IIIA, IIIB, or metastatic breast cancer who met eligibility criteria were enrolled between May 1996 and January 1998 (Table 1). All patients were required to have a good performance status and adequate cardiac, pulmonary, renal, and hepatic function. Women with active, untreated central nervous system metastases were not eligible, nor were women with >5% of the bone marrow cellularity histologically involved with breast cancer. Once the maximum tolerated dose (MTD) of IL-2 had been established, 15 consecutive women meeting the same eligibility criteria were enrolled between January and June 1998 and underwent mobilization with G-CSF alone, and thus served as a control group of patients. The protocol received institutional review board approval, and all patients gave written informed consent.

Patients with metastatic disease underwent cytoreductive salvage therapy with a regimen individualized depending on prior treatment history. Patients typically received two to three cycles of salvage chemotherapy and were eligible for study if they achieved either a CR or PR, where CR was defined as the disappearance of all measurable or evaluable tumors, and PR was defined as a 50% reduction in the size of a measurable lesion or lesions based on the sum of the products of the greatest tumor diameter and its perpendicular. For patients with bone metastases only, PR was defined as sclerosis in the affected sites without new lesions identified on reevaluation, and CR was defined by complete resolution of osseous metastases by bone scan and plain radiographs. Patients with locally advanced stage IIIA or IIIB disease received neoadjuvant chemotherapy, followed by mastectomy and axillary node dissection.

Table 1. Patient characteristics

Mobilization regimen	IL-2 + G-CSF			G-CSF
IL-2 dose ($\times 10^6$ IU/m²)	0.25–1.25	1.75	2.25	None
Number of patients	11	29	3	15
Median age	46	45	44	44
[y (range)]	(38–55)	(23–58)	(42–46)	(34–60)
Stage of disease				
IIIA/IIIB	4	6	1	4
Recurrent/metastatic	7	23	2	11
Prior chemotherapy				
Adjuvant	9	19	3	10
For metastatic disease	7	23	1	10
Prior radiation therapy				
Breast/chest wall	3	8	0	7
Other	3	2	0	0
Dominant site of disease				
Bone/bone marrow ± soft tissue	1	9	1	7
Soft tissue/lymph nodes only	6	12	2	6
Visceral ± other	4	8	0	2

PBPC mobilization and harvest

PBPC mobilization began ≥21 days following the last cycle of chemotherapy when the total white blood cell count had recovered to ≥2500/mm³ and the absolute neutrophil count to ≥1500/mm³. In patients with stage III disease, mobilization typically began 6 weeks following surgery. IL-2 (provided by Chiron, Emeryville, CA) was administered subcutaneously days 1–14 in a dose-escalated manner. Acetaminophen 650 mg, ibuprofen 400 mg, and diphenhydramine 50 mg were given to all patients as oral premedications to IL-2, to be repeated 4 hours following injection. IL-2 was tested at doses between 0.25 and 2.25 $\times 10^6$ IU/m²/day (Table 2), within a range previously reported to expand NK cells in vivo without significant toxicity [11]. Patients were not enrolled to the next dose level until a minimum of two patients had been treated at a given level with no dose-limiting toxicity (DLT) and engraftment had occurred following high-dose chemotherapy. Toxicity was formally assessed on days 7 and 14 of IL-2 administration. DLT was defined by the Cancer and Leukemia Group B expanded common toxicity criteria as grade 3 toxicity attributable to IL-2 administration. IL-2 was discontinued in patients experiencing DLT,

Table 2. Outline of interleukin 2 dose escalation

Dose of IL-2 ($\times 10^6$ IU/m²/day)	Enrolled	Underwent transplantation
0.25	5	4*
0.75	3	3
1.25	3	3
1.75	29 (8 + 21)	27†
2.25	3	2‡
Control	15	15

*One patient developed progressive disease after priming and was removed from study.

†One patient with an inadequate number of peripheral blood progenitor cells collected and marrow disease precluding marrow harvest was removed from study; one patient withdrew after requiring both peripheral blood stem cells and marrow harvest.

‡One patient withdrew from study near completion of priming and before apheresis.

then restarted at the next lower dose level once toxicity had resolved. The MTD of IL-2 was defined as the dose below that in which 33% of patients experienced DLT. Eight patients were enrolled at 1.75×10^6 IU/m²/day during the dose-escalation phase of the study. Once DLT was established at 2.25×10^6 IU/m²/day, an additional 21 patients were enrolled at 1.75×10^6 IU/m²/day to establish this dose as the MTD.

G-CSF 5 μg/kg/day was administered subcutaneously days 8–14 of the mobilization regimen, with 10- to 12-L apheresis using a CS-3000 cell sorter (Baxter, Deerfield, IL) on days 13–15. If necessary, G-CSF and aphereses were extended for up to 3 additional days to achieve the minimum required total collection of 1.5×10^6 $CD34^+$ cells/kg. If the $CD34^+$ cell number was deficient, G-CSF and IL-2 were discontinued and G-CSF priming alone was performed following a 2-week interval. If sufficient numbers of PBPC cells still were not obtained following G-CSF priming alone, bone marrow harvest was performed if the bone marrow was histologically free of tumor. Patients in the control group received the same dose of G-CSF on days 1–7 with aphereses on days 6–8, and extension of G-CSF and aphereses up to 3 additional days if required.

High-dose chemotherapy and transplantation

High-dose chemotherapy included cyclophosphamide 1500 mg/m² intravenously over 2 hours days −6, −5, −4, and −3 (total dose 6000 mg/m²), etoposide 400 mg/m² intravenously over 4 hours immediately following cyclophosphamide on days −6, −5, and −4 (total dose 1200 mg/m²), carboplatin 200 mg/m² intravenously over 1 hour immediately following etoposide on days −6, −5, and −4, and immediately following cyclophosphamide on day −3 (total dose 800 mg/m²), and thiotepa given by continuous intravenous infusion at 125 mg/m² over 24 hours days −6, −5, −4, abd −3 (total dose 600 mg/m²). All doses of chemotherapy were based on actual body surface area. MESNA (sodium 2-mercaptoethane sulfonate) and vigorous hydration were given as prophylaxis for hemmorhagic cystitis. Following 2 days of rest, all PBPCs were reinfused on day 0. G-CSF was begun on the evening of day 0 at a dose of 5 μg/kg/day given subcutaneously or as a bolus intravenous injection. G-CSF was continued until the absolute neutrophil count was ≥2500/mm³ on each of 2 consecutive days. Posttransplant radiation therapy was given to prior sites of disease involvement in patients with metastatic disease where feasible, and to the chest wall and regional lymph nodes in patients with stage III disease. Patients whose tumors were hormone receptor positive received posttransplant tamoxifen 10 mg orally twice daily.

Phenotype and cytotoxicity

Peripheral blood or a sample of the PBPC product was obtained and mononuclear cells (MNCs) prepared by Ficoll-Hypaque (specific gravity 1.077) (Sigma, St. Louis, MO) density gradient centrifugation. Cell surface antigens were determined by direct staining of cells with mouse monoclonal antibodies. Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-coupled antibodies (Becton Dickinson, Mountain View, CA) were directed at CD3, CD25, CD56, and HLA-DR. FITC- and PE-coupled isotype matched imunoglobulins were used as controls. All analyses were performed with a FACS-Calibur (Becton Dickinson) and CELLQuest software (Becton Dickinson). Cytotoxicity assays were performed in triplicate using fresh MNC against K562 (American Tissue Culture Collection) and overnight IL-2–activated (1000 IU/mL) MNC against the MCF-7 (American Tissue Culture Collection) cell lines in a 4-hour ^{51}Cr release assay. One lytic unit (LU) was defined as the number of effectors required to lyse 30% of targets; cytotoxicity is presented as LU per 10^6 effector cells [12]. To control for target variability, frozen targets (expanded from the same batch) were thawed every 4–6 weeks.

Statistics

Due to violation of assumptions, statistical comparisons of cell doses and time to engraftment between groups were performed using the Wilcoxon rank sum and Kruskal-Wallis tests of significance. Results of experimental points are reported as mean ± 1 SEM. Statistical comparisons of experimental points between independent groups were completed with the two-sided Student's *t*-test. Comparisons of values at different time points but within the same population were made with a two-sided paired-comparison *t*-test.

Results

Clinical tolerability of IL-2 mobilization

Forty-three patients received IL-2 at five dose levels + G-CSF. Fifteen control patients received G-CSF alone (Table 2). Grade I toxicity at the dose levels of 0.25 to 1.25×10^6 IU/m²/day consisted of mild fever (37.1–38.0°C) in one patient, and mild chills and myalgia in one additional patient. At the dose level of 1.75×10^6 IU/m²/day, every patient experienced grade I toxicities, primarily mild fever, chills, arthralgias, sweats, and malaise. A total of 17 patients also experienced grade II toxicities, primarily consisting of fever (38.1–40.0°C) and chills. Two patients developed thrombocytopenia (50,000–74,900/mm³), with one of these patients receiving only 10 of 14 scheduled doses of IL-2 secondary to the thrombocytopenia. One patient who developed nausea and vomiting with hypokalemia received 12 of 14 scheduled doses of IL-2. All patients at all IL-2 dose levels experienced mild erythema and induration at the injection sites.

Each of the three patients who initiated IL-2 injections at the dose level of 2.25×10^6 IU/m²/day experienced DLT. Toxicity in the first patient at this dose level consisted of fever (>40°C), pronounced and prolonged chills, and edema with weight gain of 3 kg following seven doses of IL-2. IL-2 was discontinued, and the patient completed priming with G-CSF alone. Toxicity in the second patient was characterized by intolerable malaise, fatigue, and myalgias after the first dose of IL-2. The IL-2 dose was decreased to 1.75×10^6 IU/m²/day, which was well tolerated for the duration of IL-2 priming. The third patient, who received six doses at 2.25×10^6 IU/m²/day, developed a diffuse, erythematous rash over 90% of the body surface area, weight gain of 2.9 kg with peripheral edema, fatigue, nausea, and emesis. IL-2 was held for 2 days with resolution of toxicity, then restarted at 1.75×10^6 IU/m²/day. After five additional doses of IL-2, the patient withdrew from the study and did not undergo apheresis or transplantation.

$CD34^+$ content of PBPC collections

The minimum total collection of 1.5×10^6 $CD34^+$ cells/kg was achieved following three initial aphereses in 22 (52%) of

Table 3. Number of peripheral blood progenitor cell apheresis collections by dose of interleukin 2

Dose of IL-2 ($\times 10^6$ IU/m^2/day)	Patients undergoing aphereses	Adequate after three aphereses	Required additional aphereses	Repeated G-CSF priming	Required BM harvest
0.25	5	2	2	1	—
0.75	3	2	1	—	—
1.25	3	2	1	—	—
1.75	29	14	5	6	3*
2.25	2†	2	—	—	—
Control	15	14	1	—	—

*One additional patient had inadequate CD34$^+$ collections, but bone marrow (BM) involvement with tumor precluded BM harvest.
†One patient withdrew from study near completion of priming and before apheresis.

42 patients undergoing collections after priming with IL-2 + G-CSF (Table 3). When the target number of CD34$^+$ cells was not achieved with three aphereses yet the target number was felt to be achievable with up to three additional consecutive collections, this was advised and was successful in an additional nine patients. Thus, 31 (74%) of 42 patients primed with IL-2 + G-CSF had an adequate number of CD34$^+$ cells following the initial aphereses. In the 11 patients who did not, following an interval delay of up to 14 days, repeat priming with G-CSF alone resulted in seven patients achieving adequate numbers of CD34$^+$ cells. Three of the remaining four patients underwent bone marrow harvesting; harvest was precluded in one patient who had marrow involvement with tumor.

Of the 15 control patients who were primed with G-CSF alone, 14 (93%) of 15 achieved the target number of CD34$^+$ cells following three aphereses; the remaining patient achieved the target with additional consecutive collections.

The total number of CD34$^+$ cells and MNC collected following the initial three aphereses were compared. Patients who received IL-2 at the MTD of 1.75×10^6 IU/m^2/day with G-CSF for mobilization had fewer CD34$^+$ cells compared with patients mobilized with G-CSF alone (median 1.5×10^6 cells/kg vs 3.0×10^6 cells /kg) ($p < 0.01$) and fewer MNCs (median 6.9×10^8 cells/kg vs 9.3×10^8 cells/kg) ($p = 0.02$).

Engraftment of IL-2–mobilized PBPC

Patients who received low doses of IL-2 (0.25–1.25×10^6 IU/m^2/day) with G-CSF for mobilization demonstrated a median (range) hematologic recovery of platelets >20,000/mm^3 at day 13 (8–16 days) and absolute neutrophil count >500/mm^3 at day 10 (10–12 days), whereas patients who received IL-2 at the MTD of 1.75×10^6 IU/m^2/day with G-CSF demonstrated platelet recovery at day 14 (8–68 days) and neutrophil recovery at day 10 (9–14 days). This was similar to hematologic recovery in the 15 patients mobilized with G-CSF alone who achieved platelets >20,000/mm^3 at day 12 (10–20 days) ($p = 0.12$) and absolute neutrophil count >500/mm^3 at day 10 (8–21 days) ($p = 0.26$).

Graft phenotype and cytolytic function

Blood MNCs were studied from patients prior to mobilization (baseline), after 7 days of IL-2 but before initiation of G-CSF and from the PBPC product. Cells were tested in cytotoxicity assays without further exogenous activation against the K562 tumor target as a measure of NK cell function (Fig. 1). In agreement with our previous studies on the effect of G-CSF on normal NK cells [10], NK cells from the PBPC product of patients mobilized with G-CSF alone exhibited less cytotoxicity compared to their baseline ($p = 0.014$, Fig. 1A). NK cells from the PBPC product of patients mobilized with G-CSF and 0.25–1.25×10^6 IU/m^2/day IL-2 also exhibited a trend toward less cytotoxicity, but not at statistically significant levels (Fig. 1B). In contrast, mobilization with 1.75×10^6 IU/m^2 of IL-2 with G-CSF prevented the decrease in NK cell function induced by G-CSF and resulted in significantly greater ($p = 0.003$) lytic function, as potent as that seen on day 7 IL-2/pre-G-CSF (Fig. 1C). There also was significantly greater cytotoxicity ($p = 0.022$) in the PBPC product mobilized with 1.75×10^6 U/m^2 IL-2 and G-CSF compared to the PBPC product mobilized with G-CSF alone.

Similar studies were performed to assess the effect of mobilization with IL-2 and G-CSF in cytotoxicity assays against the breast cancer cell line MCF-7. Evaluation of cytotoxicity without further exogenous IL-2 activation resulted in low levels of lytic activity from all time points (data not shown), consistent with the need for further induction of lytic machinery by IL-2 for lysis of this NK resistant target [13]. Cytotoxicity was significantly higher compared to baseline when cells were taken after 7 days of IL-2/pre-G-CSF ($p = 0.006$) or from the PBPC product ($p = 0.001$) of patients mobilized with both 1.75×10^6 IU/m^2/day IL-2 and G-CSF (Fig. 2C), but not in patients mobilized with 0.25–1.25×10^6 IU/m^2/day IL-2 and G-CSF (Fig. 2B).

The composition of lymphocytes within the PBPC products or the peripheral blood was evaluated by immunophenotyping (Fig. 3). The percentage of T cells and the CD4:CD8 ratios were similar between PBPC mobilized with G-CSF alone or with the addition of IL-2 (p = NS, data not shown). In contrast, a higher percentage of NK cells was present in PBPC mobilized with high dose IL-2 (1.75×10^6 IU/m^2/day) + G-CSF compared with baseline ($p = 0.015$) than the group receiving G-CSF alone (p = NS) (Fig. 3A). There was no difference in the number of T cells expressing HLA-DR or CD25 from products of patients not

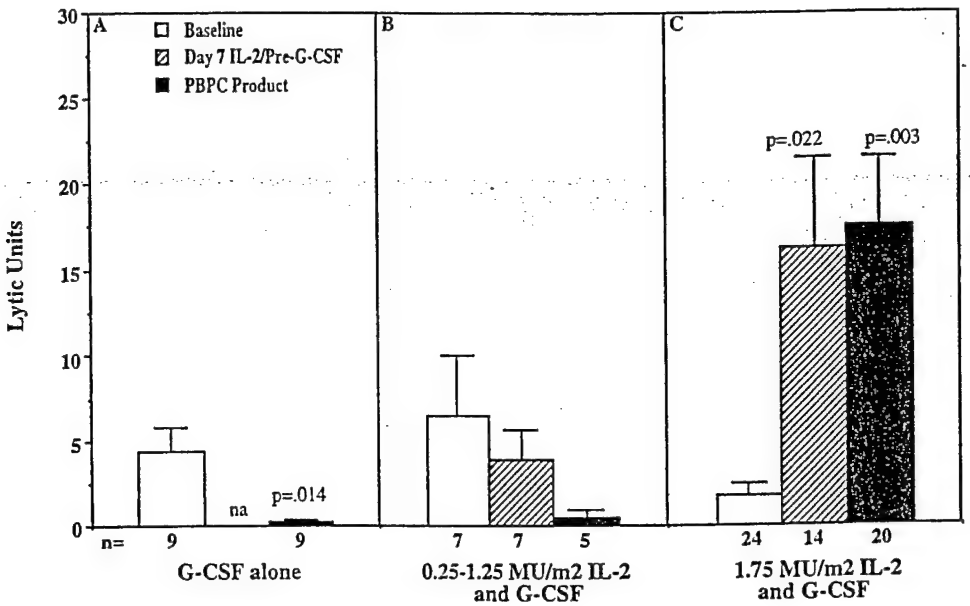

Figure 1. Addition of subcutaneous interleukin 2 (IL-2) to granulocyte colony-stimulating factor (G-CSF) mobilization reverses G-CSF induced natural killer cell suppression. Mononuclear cells from the peripheral blood or peripheral blood progenitor cells (PBPC) from patients who received G-CSF alone (A), 0.25–1.25 × 10^6 IU/m^2 IL-2 and G-CSF (B), or 1.75 × 10^6 IU/m^2 and G-CSF (C) were tested without exogenous IL-2 activation for cytotoxicity against chromium-labeled K562 targets prior to mobilization (baseline sample) on day 7 of IL-2/pre-G-CSF or from the PBPC product. Data are presented as mean ± SEM of the average of triplicate wells from each population expressed in lytic units. Comparisons were made to the baseline samples, and significant p values shown. n = number of samples; na = not available.

receiving IL-2 compared with the baseline sample, although the number of T cells that were HLA-DR positive early after engraftment may be slightly higher than pretransplant in those patients ($p = 0.08$ in six paired samples, Fig. 3B). In contrast, larger fractions of T cells expressing HLA-DR or CD25 could be found in products mobilized from patients receiving high-dose IL-2 (1.75 × 10^6 IU/m^2/day) and G-CSF ($p = 0.004$ or 0.006 respectively, Fig. 3B–C) compared to the baseline sample.

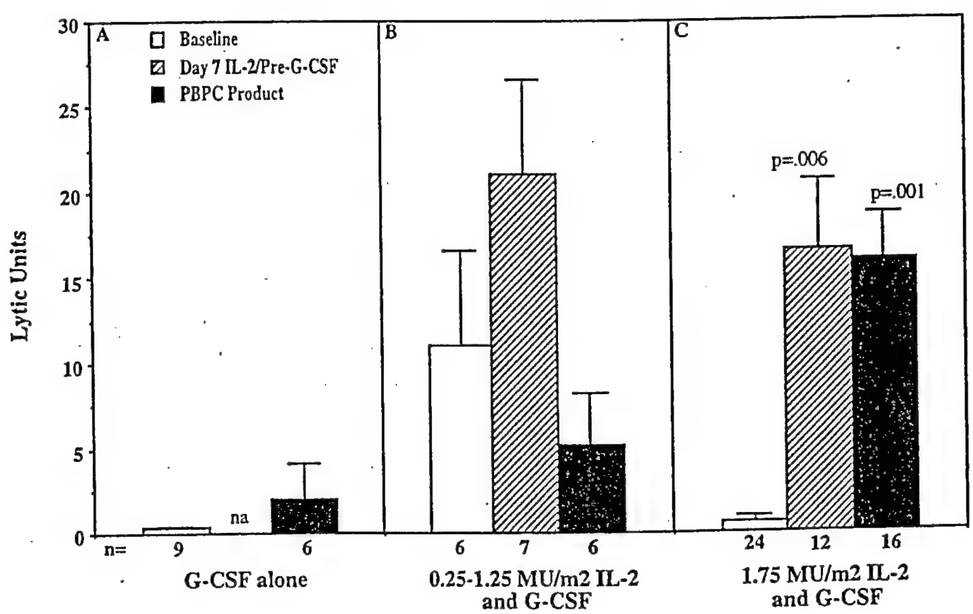

Figure 2. Addition of subcutaneous interleukin 2 (IL-2) to granulocyte colony-stimulating factor (G-CSF) mobilization increases IL-2 activation and cytotoxicity against breast cancer targets. Mononuclear cells from the peripheral blood or peripheral blood progenitor cells (PBPC) from patients who received G-CSF alone (A), 0.25–1.25 × 10^6 IU/m^2 IL-2 and G-CSF (B), or 1.75 × 10^6 IU/m^2 and G-CSF (C) were incubated overnight in serum-free media supplemented with 1000 IU/ml IL-2 and then tested against chromium-labeled MCF-7 breast cancer targets prior to mobilization (baseline sample) on day 7 of IL-2/pre-G-CSF or from the PBPC product. Data are presented as mean ± SEM of the average of triplicate wells from each population expressed in lytic units. Comparisons were made to the baseline samples, and significant p values shown. n = number of samples; na = not available.

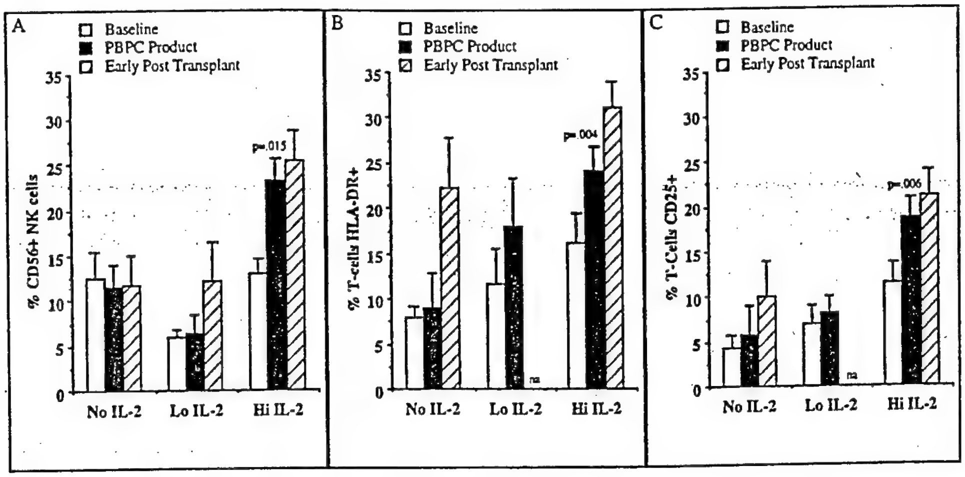

Figure 3. Addition of interleukin 2 (IL-2) to granulocyte colony-stimulating factor (G-CSF) mobilization increases the number of natural killer (NK) cells and activated T cells in the peripheral blood progenitor cell (PBPC) product. Increased NK cell number is sustained in blood early posttransplant. Mononuclear cells were obtained from the peripheral blood or PBPC product from patients who received only G-CSF (No IL-2), 0.25–1.25 × 10^6 IU/m^2 IL-2 and G-CSF (Lo IL-2), or 1.75 × 10^6 IU/m^2 IL-2 and G-CSF (Hi IL-2). The percentage of CD56$^+$ NK cells (A), CD3$^+$/HLA-DR$^+$ T-cells (B), and CD3$^+$/CD25$^+$ T cells (C) was determined from blood prior to mobilization (baseline, white bars), from the PBPC product (black bars), or from the patients' blood collected between 7 and 14 days after transplant (hatched bars). Comparisons were made to the baseline samples, and significant p values shown. na = not available.

The enhanced NK cell number in the PBPC graft was sustained in the first 2 weeks posttransplant. Blood mononuclear cells collected from patients 7–14 days after transplant contained significantly more NK cells ($p = 0.047$) when patients received an IL-2 (1.75 × 10^6 IU/m^2/day) and G-CSF mobilized graft compared to patients who received a graft mobilized with G-CSF alone (Fig. 3A). This difference were not sustained 21–28 days after transplantation. Despite the enhanced NK cell number early posttransplant in those patients who received an IL-2 + G-CSF mobilized graft, corresponding cytotoxicity of mononuclear cells was low against K562 (3.9 ± 2.7 lytic units, n = 10) and MCF-7 (0.6 ± 0.3 lytic units, n = 10) even after further ex vivo IL-2 incubation. The percentage of activated T cells in the early posttransplant period was similar between groups.

Conclusion

This trial tested the combination of subcutaneous IL-2 with G-CSF in mobilization of PBPC and evaluated the engraftment and immune recovery posttransplantation. Toxicity of IL-2 clearly was dose related. DLT was noted at the 2.25 × 10^6 IU/m^2/day dose level and quickly subsided with discontinuation of IL-2 therapy. The MTD in this trial was 1.75 × 10^6 IU/m^2/day. Although well tolerated at this dose level, 17 (57%) of 29 patients experienced grade II toxicities that, in all but two patients, did not preclude completion of the full course of IL-2.

IL-2 used for mobilization does not have a deleterious effect on the graft's ability to achieve timely hematopoietic reconstitution in the host. However, use of IL-2 clearly modified the efficiency of CD34$^+$ collection. As the dose of IL-2 increased, the number of CD34$^+$ cells and the total MNCs collected in three aphereses decreased. Approximately half of the patients treated with IL-2 (1.75–2.25 × 10^6 IU/m^2/day) required additional aphereses or rarely a bone marrow harvest to obtain the minimum number of CD34$^+$ cells satisfactory to proceed with transplantation, thus not only increasing the associated expense of additional procedures but also potentially increasing the likelihood of tumor cell contamination in the collected stem cell product [14]. The need for additional mobilization in patients treated with IL-2 as compared with the control group cannot be attributed to differences in prior therapy, as the groups were quite similar in this regard (Table 1). The 5 μg/kg/dose of G-CSF and timing of aphereses used in our study were chosen, as they were those used in an intergroup breast cancer trial in which our institution was participating at the time this study was initiated. Since then, a dose-response effect has been demonstrated for G-CSF [15,16], and the timing of apheresis has been shown to affect the quantity of harvested stem cells [17]. It is conceivable that by increasing the dose of G-CSF or varying the timing of aphereses, a greater number of patients who received IL-2 would not have needed to undergo additional aphereses or bone marrow harvest.

One other group has a preliminary report utilizing IL-2 with G-CSF for stem cell mobilization. Sosman et al. [18] used Amgen IL-2 given intravenously for 96 hours/week × 2 with G-CSF at 10 μg/kg/day for 7 days in patients with breast cancer, followed by high-dose chemotherapy and autotransplantation. Patients mobilized with IL-2 + G-CSF had fewer CD34$^+$ cells harvested compared to those given G-CSF alone, similar to our data.

The mechanism of how IL-2 decreases progenitor cell mobilization is not known. Homing of stem cells likely involves interactions between adhesion receptors on cells and

ligands on stroma. Although the mobilization process likely is complex, it is presumed to result from changes in adhesive interactions in the marrow microenvironment leading to egress of progenitors into the blood [19]. IL-2 has been shown to alter the growth of hematopoietic progenitors in long-term culture where IL-2 had no direct effect on colony growth itself, suggesting a modulating effect through mesenchymal stromal elements [20]. Alternatively, IL-2 effects may be indirect through release of secondary cytokines such as interferon or transforming growth factor β. Interferon has been shown to increase adhesion of chronic myelogenous leukemia progenitors to stroma [21]. Therefore, IL-2 may lead to direct or indirect increased adhesion to marrow stroma, preventing the normal release of stem cells expected with G-CSF mobilization. Further studies will be needed to establish this mechanism.

IL-2–mobilized grafts showed enhanced immunologic function. MNCs from the PBPC product of patients mobilized with IL-2 + G-CSF demonstrate enhanced cytotoxicity against K562 and MCF-7 targets compared with cells from patients mobilized with G-CSF alone. IL-2 also reversed G-CSF–induced NK suppression. The increased cytotoxicity of the MNCs from the IL-2 + G-CSF mobilized PBPC product may be underestimated by the results reported here, as we previously demonstrated suppression induced by the apheresis procedure itself, consistent with results reported by others [22]. The ultimate test of increased NK cytotoxicity against a tumor cell would be demonstration of NK cell lysis of autologous tumor cells. However, isolation of primary breast cancer cells for use in in vitro cytotoxicity assays remains technically difficult and problematic.

IL-2 mobilization increased the number of NK and activated T cells in the PBPC graft. In their study, Sosman et al. [18] noted that IL-2 + G-CSF mobilized more activated T cells, NK cells, and activated NK cells compared to G-CSF alone, and engraftment of seven patients treated at the lowest dose of IL-2 (1.8 × 10^6 IU/m^2/day) compared favorably with patients mobilized with G-CSF alone, all consistent with our data although with a different dose, schedule, and route of IL-2 administration. Although IL-2 mobilization resulted in a higher level of NK cells during the first 2 weeks posttransplantation, corresponding cytotoxicity of MNCs was low against both K562 and MCF-7 targets even after further ex vivo IL-2 incubation. This is explained, at least in part, by the posttransplant G-CSF administration all patients received until neutrophil engraftment. The suppressive effect of G-CSF administration after transplant may abrogate the increased NK cell function found in the PBPC graft itself. Therefore, additional immunotherapy beginning in the early posttransplant period may be required to further enhance the number and function of NK cells and other lymphoid effectors.

Other trials reported investigating IL-2 as an approach to immunotherapy in conjunction with high-dose chemotherapy and autografting [23–29]. Most trials used infusional IL-2 initiated following engraftment, due to the concern about possible IL-2–induced delay in hematopoietic reconstitution. We and others reported using IL-2 in the immediate posttransplant time period. We treated ALL autograft recipients with IL-2 by continuous infusion 4 days/week during the first 4 weeks posttransplant [28]. Engraftment was timely and immunologic activation was modest, but fever and weight gain were dose limiting. Lister et al. [29] treated autograft patients (11 with relapsed lymphoma and one with metastatic breast cancer) on day 2 after transplant with infusion of ex vivo cultured, autologous activated NK cells, with continuous infusion IL-2 (Chiron, 2 × 10^6 IU/m^2/day), followed by a 90-day continuous infusion at 3 × 10^5 IU/m^2/day. All patients engrafted, and nine completed treatment. Overall toxicity associated with early posttransplant transfer of activated NK cells and IL-2 was tolerable and similar to control transplant patients. Meehan et al. [30] cultured the PBPC product in IL-2 (Chiron) for 24 hours before infusion followed by parenteral IL-2 in a dose-escalating manner beginning the day after autologous transplantation. The IL-2–activated PBPC contained an increased percentage of CD3$^+$,CD25$^+$, HLA$^-$DR$^-$ T cells. All patients engrafted, but one of three patients was unable to complete the planned course of posttransplant IL-2 secondary to toxicities of fever, fatigue, and weight gain.

We conclude that subcutaneous IL-2 can be given safely in conjunction with G-CSF to mobilize PBPC. Our results demonstrate that IL-2 + G-CSF may be an effective way to enhance the number and function of anti-tumor effector cells within an autograft without compromising hematologic recovery. A major limitation to the use of IL-2 for priming is the decrease in number of CD34$^+$ cells mobilized, a limitation that theoretically may be overcome by an increased dose of G-CSF and/or timing of stem cell collections. In addition, the duration of the enhanced graft-vs-tumor effect mediated by the IL-2 + G-CSF mobilized graft is short, signifying the need for additional posttransplant immunotherapy to maintain and further enhance anti-tumor effector cell function. Further study combining IL-2 graft activation with posttransplant immunotherapy is needed to determine the clinical efficacy of this approach in preventing posttransplant tumor recurrence.

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References

1. Antman KH, Rowlings PA, Vaughan WP, Pelz CJ, Fay JW, Fields KK, Freytes CO, Gale RP, Hillner BE, Holland K, Kennedy MJ, Klein

JP, et al (1997) High-dose chemotherapy with autologous hematopoietic stem-cell support for breast cancer in North America. J Clin Oncol 15:1870

2. Laport GF, Grad G, Grinblatt DL, Bitran JD, Williams SF (1998) High-dose chemotherapy consolidation with autologous stem cell rescue in metastatic breast cancer: a 10 year experience. Bone Marrow Transplant 21:127
3. Ayash LJ, Wheeler C, Fairclough D, Schwartz G, Reich E, Warren D, Schnipper L, Antman K, Frei E 3rd, Elias A (1995) Prognostic factors for prolonged progression-free survival with high-dose chemotherapy with autologous stem-cell support for advanced breast cancer. J Clin Oncol 13:2043
4. Patrone F, Ballestero A, Ferrando F, Brema F, Moraglio L, Valbonesi M, Basta P, Ghio R, Gobbi M, Sessarego M (1995) Four-step high-dose sequential chemotherapy with double hematopoietic progenitor-cell rescue for metastatic breast cancer. J Clin Oncol 13:840
5. Bitran JD, Samuels B, Klein L, Hanauer S, Johnson L, Martinec J, Harris E, Kempler J, White W (1996) Tandem high-dose chemotherapy supported by hematopoietic progenitor cells yields prolonged survival in stage IV breast cancer. Bone Marrow Transplant 17:157
6. Tallman MS, Rademaker AW, Jahnke L, Brown SG, Bauman A, Mangan C, Kelly C, Rubin H, Kies MS, Shaw J, Kiel K, Gordon LI, et al (1997) High- dose chemotherapy, autologous bone marrow or stem cell transplantation and post-transplant consolidation chemotherapy in patients with advanced breast cancer. Bone Marrow Transplant 20:721
7. deMagalhaes-Silverman M, Hammert L, Lembersky B, Lister J, Rybka W, Ball E (1998) High-dose chemotherapy and autologous stem cell support followed by post-transplant doxorubicin and taxol as initial therapy for metastatic breast cancer: hematopoietic tolerance and efficacy. Bone Marrow Transplant 12:1207
8. Kennedy MJ, Jones RJ (1993) Autologous graft-versus-host disease: immunotherapy of breast cancer after bone marrow transplantation. Breast Cancer Res Treat 26:S-31
9. Or R, Ackerstein A, Nagler A, Kapelushnik J, Naparstek E, Samuel S, Amar A, Bruatbar C, Slavin S (1998) Allogeneic cell-mediated immunotherapy for breast cancer after autologous stem cell transplantation: a clinical pilot study. Cytokines Cell Mol Ther 4:1
10. Miller JS, Prosper F, McCullar V (1997) Natural killer cells (NK) are functionally abnormal and NK progenitors are diminished in G-CSF mobilized peripheral blood progenitor cell collections. Blood 90:3098
11. Meropol NJ, Porter M, Blumenson LE, Lindemann MJ, Perez RP, Vaickus L, Loewen GM, Creaven PJ, Wilkes KA, Giedlin MA, Caligiuri MA (1996) Daily subcutaneous injection of low-dose interleukin 2 expands natural killer cells in vivo without significant toxicity. Clin Cancer Res 2:669
12. Katsanis E, Anderson PM, Filipovich AH, Hasz DE, Rich ML, Loeffler CM, Ochoa AC, Weisdorf DJ (1991) Proliferation and cytolytic function of anti-CD3 + interleukin-2 stimulated peripheral blood mononuclear cells following bone marrow transplantation. Blood 78:1286
13. Miller JS, Tessmer-Tuck J, Pierson BA, Weisdorf D, McGlave P, Blazar BR, Katsanis E, Verfaillie C, Lebkowski J, Radford JR Jr, Burns LJ (1997) Low dose subcutaneous interleukin-2 after autologous transplantation generates sustained in vivo natural killer cell activity. Biol Blood Marrow Transplant 26:287
14. Kahn DG, Prilutskaya M, Cooper B, Khan DG, Prilutskaya M, Cooper B, Kennedy MJ, Meagher R, Pecora AL, Petri RA, Moreb J, Wingard J, Rosenfeld C, Herzig RM, Glassco JE, et al. (1997) The relationships between the incidence of tumor contamination and number of phereses for stage IV breast cancer. Blood 90(suppl.):565a.
15. Erban J, Miller K, Berkman E, Weckstein D, Coury P, Sweet M, Schenkein D (1995) Filgrastim priming of PBSC and hematopoietic reconstitution following high dose chemotherapy for breast cancer: effect of dose on PBSC yield and engraftment. Proc Am Soc Clin Oncol 14:929
16. Kroger N, Zeller W, Hassan HT, Wruger W, Gutensohn K, Loliger C, Zander AR (1999) Stem cell mobilization with G-CSF alone in breast cancer patients: higher progenitor cell yield by delivering divided doses (2 × 5 μg/kg) compared to a single dose (1 × 10 μg/kg). Bone Marrow Transplant 23:125
17. Knudsen LM, Gaarsdal E, Jensen L, Nikolaisen K, Johnsen HE (1998) Evaluation of mobilized CD34+ cell counts to guide timing and yield of large-scale collection by leukapheresis. J Hematother 7:45
18. Sosman J, Moss ST, Martone B, Bayer R, Peace D, Stock W, Stiff PJ (1996) IL-2 plus G-CSF for peripheral cell mobilization in breast cancer patients receiving high dose chemotherapy: preliminary results reveal marked increases in activated T and NK cells in the mobilized stem cell source, while subsequent hematologic recovery following chemotherapy remains rapid (abstr). Blood 88(suppl. 1):642a
19. Prosper F, Stroncek D, Verfaillie CM (1996) Phenotypic and functional characterization of long-term culture-initiating cells present in peripheral blood progenitor collections of normal donors treated with granulocyte-stimulating factor. Blood 88:2033
20. Cashman JD, Eaves AC, Raines EW, Ross R, Eaves CJ (1990) Mechanisms that regulate the cell cycle status of very primitive hematopoietic cells in long-term human marrow cultures. I. Stimulatory role of a variety of mesenchymal cell activators and inhibitory role of TGF-beta. Blood 1:96
21. Bhatiz R, McCarthy J, Verfaillie CM (1996) Interferon-alpha restores normal beta 1 integrin-mediated inhibition of hematopoietic progenitor proliferation by the marrow microenvironment in chronic myelogenous leukemia. Blood 87:3883
22. Talmadge JE, Reed EC, Kessinger A, Kuszynski CA, Perry GA, Cordy CL, Mills KC, Thomas ML, Pirruccello SJ, Letheby BA, Arneson MA, Jackson JD (1996) Immunologic attributes of cytokine mobilized peripheral blood stem cells and recovery following transplantation. Bone Marrow Transplant 17:101
23. Higuchi CM, Thompson JA, Petersen FB, Buckner CD, Fefer A (1991) Toxicity and immunomodulatory effects of interleukin-2 after autologous bone marrow transplantation for hematologic malignancies. Blood 77:2561
24. Blaise D, Olive D, Stoppa AM, Viens P, Pourreau C, Lopez M, Attal M, Jasmin C, Monges G, Mawas C, Mannoni P, Palmer P, et al (1990) Hematologic and immunologic effects of the systemic administration of recombinant interleukin-2 after autologous bone marrow transplantation. Blood 76:1092
25. Benyunes MC, Higuchi C, York A, Lindgren C, Thompson JA, Buckner CD, Fefer A (1995) Immunotherapy with interleukin-2 with or without lymphokine-activated killer cells after autologous bone marrow transplantation for malignant lymphoma: a feasibility trial. Bone Marrow Transplant 16:283
26. Soiffer RJ, Murray C, Cochran K, Cameron C, Wang E, Schow PW, Daley JF, Ritz J (1992) Clinical and immunologic effects of prolonged infusion of low dose recombinant interleukin-2 after autologous and T-cell depleted allogeneic bone marrow transplantation. Blood 79:517
27. Robinson N, Benyunes MC, Thompson JA, York A, Petersdorf S, Press O, Lindgren C, Chauncey T, Buckner CD, Bensinger WI, Appelbaum FR, Fefer A (1997) Interleukin-2 after autologous stem cell transplantation for hematologic malignancy: a phase I/II study. Bone Marrow Transplant 19:435
28. Weisdorf DJ, Anderson PM, Blazar BR, Uckun FM, Kersey JH, Ramsay NK (1993) Interleukin-2 immediately after autologous bone marrow transplantation for acute lymphoblastic leukemia: a phase I study. Transplantation 55:61
29. Lister J, Tybka WB, Donnenberg AD, deMagalhaes-Silverman M, Pincus SM, Bloom EJ, Elder EM, Ball ED, Whiteside TL (1995) Autologous peripheral blood stem cell transplantation and adoptive immunotherapy with activated natural killer cells in the immediate post-transplant period. Clin Cancer Res 6:607
30. Meehan KR, Verma UN, Cahill R, Frankel S, Areman EM, Sacher RA, Foelber R, Rajagopal C, Gehan EA, Lippman ME, Mazumder A (1997) Interleukin-2-activated hematopoietic stem cell transplantation for breast cancer: investigation of dose level with clinical correlates. Bone Marrow Transplant 20:643

IL-2 Based Immunotherapy after Autologous Transplantation for Lymphoma and Breast Cancer Induces Immune Activation and Cytokine Release: A Phase I/II Trial

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Abstract

In vitro studies suggest that interleukin-2 (IL-2) activated natural killer (NK) cells mediate lytic activity against lymphoma and breast cancer targets. We determined the maximum tolerated dose (MTD) of subcutaneous IL-2 following hematopoietic recovery from autologous transplantation, as well as the safety and immune activating effects of intravenous infusion of either *ex vivo* IL-2 activated NK cells (part I of the study) or IL-2 boluses (part II of the study). Fifty-seven patients with relapsed lymphoma (n=29) or metastatic breast cancer (n=28) were enrolled. In part I of the study, 34 patients were enrolled at 4 dose levels of subcutaneous IL-2, from 0.25 -1.75 x 10^6 IU/m^2/day, self-administered for 56 consecutive days. On days 28 and 42 of subcutaneous IL-2, patients underwent lymphaphereses with overnight *ex vivo* IL-2 activation of the pheresis product. IL-2 activated cells were reinfused the following day at 3 cell dose levels, 4.0 x 10^7kg, 8.0 x 10^7kg or full product. In part II of the study, 23 patients received subcutaneous IL-2 at the established MTD, 1.75 x 10^6 IU/m^2/day, for 42 days. Patients were enrolled at 3 dose levels of supplemental intravenous IL-2 bolus infusions, 2, 4, or 6 x 10^6 IU/m^2 given on days 28 and 35 of subcutaneous IL-2 therapy, designed to further increase NK cell number and function *in vivo*. Toxicities from subcutaneous IL-2, full product infusions or the maximum dose of bolus IL-2 were generally mild. There was a 10-fold increase in circulating NK cells, which was sustained throughout the IL-2 treatment period. Prior to subcutaneous IL-2, peripheral blood mononuclear cells (PBMNC) did not have lytic activity against NK resistant targets, Raji (lymphoma) and MCF-7 (breast cancer). *In vitro* cytotoxicity was modestly enhanced following 28 days of subcutaneous IL-2. Lytic function was markedly enhanced for cells incubated overnight with IL-2 and for fresh PBMNC obtained one day post-infusion of activated cells. Post-infusion of IL-2 boluses, cytotoxicity was markedly enhanced to a degree comparable to that seen following infusion of *ex vivo* IL-2 activated cells. Circulating cytokine levels of IL-6, IFN-γ, and TNF-α were transiently but significantly increased in patients, peaking 2 hours after each IL-2 bolus infusion. To determine if this treatment strategy affected outcomes, we performed a matched pairs analysis of patients treated with the optimum dose of post-transplant immunotherapy (n=27) with matched controls from the Autologous Blood and Marrow Transplant Registry database. There were no statistically significant differences in

survival or disease-free survival. We conclude that subcutaneous IL-2 and either IL-2 activated cells or IL-2 bolus infusions can be safely administered in the outpatient setting following autologous transplantation, and generates PBMNC with enhanced cytotoxicity against NK resistant lymphoma and breast cancer targets. With this dose and schedule of administration of IL-2, no improvement in patient disease outcomes was noted, but as the power to detect a difference in this matched-pairs analysis was low due to limited patient numbers, no conclusions can be drawn regarding clinical efficacy. Further study is needed to take advantage of the immunotherapeutic potential of IL-2 activated NK cells.

Introduction

The use of high dose chemotherapy with autologous stem cell support has been widely employed in the treatment for relapsed lymphoma and metastatic breast cancer. In chemotherapy sensitive lymphoma and metastatic breast cancer patients, autologous transplantation can result in high remission rates (1, 2). However, many patients who achieve a complete response following transplantation will relapse and die of their disease. Most patients relapse at sites of previous bulk disease, suggesting that the primary reason for treatment failure is inability to eradicate chemotherapy resistant clonogenic tumor. An inadequately functioning immune system may contribute to early cases of relapse, especially in patients who fail to achieve a complete remission following autologous transplantation.

Human natural killer (NK) cells represent a population of large granular lymphocytes that express the CD56+/CD3- phenotype. In humans and animals, recombinant interleukin-2 (IL-2) stimulates both NK expansion and cytotoxic activity against various fresh and cultured tumor targets *in vivo* and *in vitro*. Autologous IL-2 activated NK cells kill a broad spectrum of tumor targets, including lymphoma, leukemia and breast cancer (3, 4). Theoretically, IL-2 activated NK cells, functioning as short-term anti-tumor therapy, would be most efficacious in a minimal residual setting such as that induced by autologous transplantation. Following initial reports in the late 1980s and early 1990s of the feasibility and immunologic potential of post-transplant IL-2 immunotherapy (5-7), many investigators explored IL-2 and/or IL-2 activated effectors for a variety of malignancies (8-25). Although these phase I/II trials have involved small numbers of clinically heterogeneous patients and different IL-2 regimens, there have been encouraging but inconclusive results. In 1997 we reported the results of a phase I trial of post-transplant IL-2 (Amgen Inc., Thousand Oaks, CA) (4). IL-2 was self-administered subcutaneously for 84 consecutive days following hematologic recovery from transplantation. Patients who received at least 28 days of IL-2 exhibited a greater than 10-fold increment in circulating CD56+bright/CD3- NK cells. Furthermore, lytic function was increased against the NK resistant targets, MCF-7 (breast) and Raji (lymphoma). Although IL-2 was able to induce immune activation *in*

vivo, the activation was submaximal. We subsequently demonstrated that NK lytic function could be further enhanced by *ex vivo* incubation of *in vivo* IL-2 primed NK cells obtained by lymphapheresis with high dose IL-2 at high cell density.

In the current study, our goal was to determine if *in vivo* primed mononuclear cells, when *ex vivo* incubated in IL-2, would generate NK cells with sustained and potent anti-tumor effects. After establishing the maximum tolerated dose (MTD) of subcutaneous IL-2 and the maximum number of mononuclear cells that could be *ex vivo* activated and safely reinfused following a single lymphapheresis, we determined the immune activating effects of IL-2 on NK cell function. We then evaluated whether intravenous boluses of IL-2 could be substituted with the same degree of safety as the *ex vivo* IL-2 activated cell infusions, while maintaining the same degree of immune activation. The effect on disease outcomes was determined by a matched pairs analysis of optimally treated patients in comparison to matched controls from the Autologous Blood and Marrow Transplant Registry (ABMTR).

Patients and Methods

Patient characteristics and eligibility criteria

Thirty-four patients between 18 and 65 years of age who had undergone an autologous stem cell transplant for non-Hodgkin's lymphoma (n = 15), Hodgkin's disease (n = 5), or metastatic breast cancer (n = 14) and had no evidence of disease progression were enrolled on the subcutanous IL-2 dose escalation and *ex vivo* IL-2 activation part of the study (Table 1). Patients with T-cell immunophenotype non-Hodgkin's disease were not eligible. Patients with Hodgkin's disease received a cytoreductive regimen of cyclophosphamide, etoposide, and BCNU (carmustine) (CBV); patients with non-Hodgkin's lymphoma received cyclophosphamide and total body irradiation unless prior radiation precluded additional radiation, in which case they received CBV (1). Patients with breast cancer received

a cytoreductive regimen of cyclophosphamide, carboplatinum, and thiotepa (26). Patients had to be transfusion independent, not requiring growth factor support, and have an unsupported platelet count $\geq$ 80,000/μL, hemoglobin $\geq$ 9 gm/dL, and an absolute neutrophil count $\geq$1000/μL. All patients were required to be at least 30 days and no later than 180 days from transplant. Twenty-three additional patients with non-Hodgkin's lymphoma (n = 5), Hodgkin's disease (n = 4), or metastatic breast cancer (n = 14) who met the same eligibility criteria were enrolled to the IL-2 bolus part of the study. The protocol received institutional review board approval and all patients exercised written informed consent when eligible for IL-2 therapy. Of the 28 patients with metastatic breast cancer, 18 (64%) also received subcutaneous IL-2 as part of pre-transplant stem cell mobilization as previously described (26).

Subcutaneous IL-2 therapy

IL-2 was provided and manufactured by Chiron (Emeryville, CA) for this study. Patients self-administered IL-2 subcutaneously for 56 days in part I of the study, and for 42 days in part II (Figure 1). Acetaminophen 650 mg, ibuprofen 400 mg, and diphenhydramine 50 mg was self-administered as oral premedications to IL-2, and repeated 4 hours following each injection. In part I of the study, subcutaneous IL-2 was tested at doses between 0.25 and 2.25 x 10^6 IU/m^2/day (Table 2), within a range previously reported to expand NK cells *in vivo* without significant toxicity (27). Patients were not enrolled to the next dose level until a minimum of 2 patients had been treated at a given level with no dose limiting toxicity (DLT) attributable to IL-2 administration. DLT was defined by the Cancer and Leukemia Group B (CALGB) expanded common toxicity criteria as Grade 3 toxicity. Toxicity was first formally assessed at day 14 of Il-2 therapy, then every 14 days until 7-14 days after IL-2 was stopped. Constitutional symptoms were assessed prospectively at each clinic visit by physician or nurse directed questionnaire. If no DLT had occurred at day 14, patients were eligible for dose escalation to the next dose level for the duration of therapy. IL-2 was discontinued in patients experiencing DLT, then restarted at the next lower dose level once toxicity had resolved. The MTD of

IL-2 was defined as the dose below that in which 33% of patients experienced DLT. The MTD was used as the daily subcutaneous dose of IL-2 in part II of the study.

Lymphapheresis and ex vivo IL-2 activation

Minimal laboratory values required prior to lymphapheresis included platelets > 50,000/μL, hemoglobin ≥ 9 gm/dL, absolute neutrophil count ≥1000/μL, and normal renal and liver function. Patients had to be outpatients with no active infections. Prior to the first lymphapheresis (day 28 of IL-2 therapy), patients must have received IL-2 for at least 21 of the first 28 days and for 5 of the 7 days immediately prior to the planned apheresis. Prior to the second lymphapheresis (day 42), patients must have received IL-2 for at least 28 of the first 42 days and 5 of the 7 days immediately prior to apheresis. Patients not meeting the criteria for apheresis continued on subcutaneous IL-2 alone. Ten-twelve liter leukaphereses were performed on days 28 and 42 using a CS3000 cell sorter (Baxter, Deerfield, IL). The lymphapheresis product was cultured in AIM-V serum-free medium (Gibco) supplemented with 1,000 IU/ml IL-2 in polyolefin gas permeable bags (Lifecell Tissue Culture Flask, Fenwal-Baxter) for 16 hours, a time period previously shown to result in sustained induction of cytolytic activity against MCF-7 and Raji cell targets (4). One hour prior to cell infusion and again 3-4 hours later, all patients received premedications of acetaminophen 650 mg orally, ibuprofen 400 mg orally, and diphenhydramine 50 mg intravenously. IL-2 activated lymphocytes were infused over 1-2 hours through a 170 μm blood administration filter the day following apheresis (days 29 and 43). Cell doses were based on mononuclear cells with the total cell count determined just prior to infusion. Patients were enrolled in cohorts of three patients each with dose escalation to a maximum of a full lymphapheresis product. Cohort 1 received 4.0×10^7 cells/kg and cohort 2 received 8.0×10^7 cells/kg (Table 3). If the planned cell dose was greater than the actual number of cells collected, then the dose infused was the full lymphapheresis product. Cohort 3 received a full lymphapheresis product. The median cell dose in a full lymphapheresis product was 7.8×10^7 cells/kg, with a range of $0.33 - 2.09 \times 10^8$ cells/kg. However, until the subcutaneous IL-2 dose escalation was completed and the MTD of

daily IL-2 determined to be 1.75 x 10^6 IU/m2/day, all patients received the lowest cell dose of 4.0 x 10^7 cells/kg. Cell dose escalation then proceeded only after all patients at a given dose were evaluated through day 56 of therapy (the last day of subcutaneous IL-2) with no DLT attributable to administration of IL-2 or IL-2 activated lymphocytes. Patients received the infusions on an outpatient basis and were observed for toxicity for a minimum of 4 hours following infusion. The subcutaneous IL-2 injection was given as scheduled that evening unless infusion related symptoms had not resolved. Patients were formally assessed for toxicity the day prior to aphersis, during and following infusion of activated cells, and the day after cell infusion. DLT was defined as Grade 2 or greater toxicity by CALGB criteria except for the following where Grade 3 toxicity was dose limiting: liver, neurologic, hematologic, infection, pulmonary, dermatologic, allergy, performance status. A platelet count of < 30,000/μL was considered dose limiting. After the MTD of IL-2 in combination with a full lymphapheresis was shown to be safe, accrual was extended to both ascertain safety and until part II of the study utilizing bolus IL-2 infusions could be initiated.

Intravenous (IV) Bolus IL-2

The eligibility requirements for part II of the study were identical to part I of the study. All patients initiated subcutaneous IL-2 at the MTD of 1.75 x 10^6 IU/m^2/day. If no DLT had occurred after 14 days, the patient continued at that dose for a total of 42 days of therapy. Patients who developed intolerable constitutional symptoms that did not constitute DLT decreased their IL-2 dose to 1.25 x 10^6 IU/m^2day for the remainder of the study. Patients received the same premedications for IV boluses of IL-2 which were given over 2 hours on days 28 and 35, with dose escalation per cohort of 3 patients each of 2.0, 4.0 and 6.0 x 10^6 IU/m^2 per infusion (Table 4). The dose of bolus IL-2, however, was not escalated until all patients in a given cohort had completed the entire course of IL-2 therapy. Hence, more than 3 patients were ultimately enrolled in the second and third cohorts. Prior to receiving bolus IL-2, patients must have received IL-2 for at least 21 of the first 28 days and for 5 of 7 days immediately prior to the planned infusion. Patients not meeting these criteria continued on daily

subcutaneous IL-2 alone. The IL-2 was infused over 2 hours on an outpatient basis and patients were monitored for a minimum of 4 hours for complications. Patients were formally assessed for toxicity the day prior to the IL-2 bolus infusion, during and following the infusion and the day after infusion. The definition of DLT for bolus IL-2 was the same as for *ex vivo* IL-2 activated cell infusions.

Cytolytic activity

Heparinized peripheral blood, serum, and plasma were obtained at each clinic visit. Mononuclear cells (PBMNC) were prepared from peripheral blood by Ficoll-Hypaque (sp. grav. 1.077) (Sigma, St. Louis, MO) density gradient centrifugation as described (4). Cell surface antigens were determined by direct staining of cells with mouse monoclonal antibodies. Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-coupled antibodies (Becton Dickinson, Mountain View, CA) were directed at CD3, CD25, CD56, and HLA-DR. FITC- and PE-coupled isotype matched immunoglobulins were used as controls. All analyses were performed with a FACSCalibur (Becton Dickenson) and CELLQusest software (Becton Dickenson). Cytotoxicity assays were performed in triplicate using fresh PBMNC or 16 hour IL-2 activated PBMNC against the NK-sensitive cell line K562 (American Tissue Culture Collection [ATCC], Rockville, MD), the NK-resistant B-cell lymphoma line Raji (ATCC) and the breast cancer cell line MCF-7 (ATCC) in a four hour ^{51}Cr release assay (4). To control for target variability, frozen targets (expanded from the same batch) were thawed every 4-6 weeks.

Cytokine levels

Serum or plasma was stored at -70°C and later assayed for cytokine levels using ELISA kits for interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). Assays were performed using the manufacturer's recommendations (R&D Systems, Minneapolis, MN). Normal cytokine values for > 95% of healthy individuals are considered by the

University of Minnesota Cytokine Reference Laboratory to be as follows: IL-1β (< 0.9 pg/mL), IL-6 (< 3.0 pg/mL), TNF-α (< 3.0 pg/mL), and IFN-γ (< 3.0 pg/mL).

Statistics

Results of experimental points were reported as mean ± standard error of the mean (S.E.M.). Statistical comparisons of experimental points between independent groups were completed with the two-sided Student's t-test. Comparisons of values at different time points but within the same population were made with a two-sided paired-comparison t-test.

The matched pairs analysis was conducted using a maximum of three controls from the ABMTR for each patient receiving IL-2. Controls were selected from 9662 potentially eligible patients from the ABMTR database who had received an autologous transplant for lymphoma or breast cancer. These patients had not received any post-transplant immunotherapy and were not transplanted at one of the study institutions. Patients who were enrolled at the MTD of subcutaneous IL-2 and received at least one of the planned *ex vivo* IL-2 activated cell infusions or IL-2 boluses were matched to controls for the following: disease, disease stage and status at time of transplant, year of transplant (within 5 years), age at transplant (within 5 years), and time from diagnosis to transplant (within 1 year). Patients with breast cancer were also matched to controls for the presence of visceral metastases and hormone receptor status. For cases with more than three controls after fitting the specific matching criteria, the three that were closest in age to the patient were chosen. Controls that died or relapsed prior to the day of initiation of IL-2 therapy in their respectively matched case were excluded. Statistical significance of the use of IL-2 on the endpoints of survival and disease-free survival was based on the result of the Wald Chi-Square test from a Cox regression model after stratifying by each matched pair (28). The use of IL-2 was tested for proportional hazards prior to inclusion in the model.

Results

Clinical tolerability of subcutaneous IL-2 therapy

Thirty-four patients were enrolled to 4 dose levels of IL-2 (Table 2). All but 2 patients enrolled at the dose levels of 0.25 to 1.25 x 10^6 IU/m^2/day were able to dose escalate by one dose level at day 14 of IL-2 treatment. One patient enrolled at 0.75 x 10^6 IU/m^2/day did not dose escalate secondary to thrombocytopenia (decline from 201,000/μL to 139,000/μL after 14 days of therapy); one patient enrolled at 1.25 x 10^6 IU/m^2/day did not dose escalate because of fatigue and nausea. One patient was removed from study after completing 22 days of IL-2 at 0.25 x 10^6 IU/m^2/day after developing a skin abscess at an injection site. Toxicities were otherwise mild, including Grade I fatigue, nausea, rash, cough, fever, myalgias, and sweats. In addition, the majority of patients developed transient local skin induration and erythema around the subcutaneous injection sites, typically 1-2 cm in diameter.

A total of 25 patients began IL-2 at 1.75 x 10^6 IU/m^2/day. Ten (40%) of patients were unable to have their dose escalated at day 14 due to side effects, and 7 of these patients ultimately were removed from study. Toxicities included: severe swelling of the tongue and face (n=1), thrombocytopenia (n=2), nausea and vomiting (n=1), intolerable fatigue (n=5), edema (n=1), and bacterial infection (n=1). Eighteen (72%) of the 25 patients who began IL-2 at 1.75 x 10^6 IU/m^2/day received ≥ 95% of the total number of planned injections. Fifteen (60%) of the 25 patients were able to dose escalate to 2.25 x 10^6 IU/m^2/day on day 14. Of these, 13 were able to remain on the higher dose for the duration of treatment, whereas the other 2 patients required a subsequent decrease back to the 1.75 x 10^6 IU/m^2/day dose level because of either anemia or thrombocytopenia. No patient required hospitalization, and all toxicity resolved within one week of discontinuation of IL-2. The MTD was 1.75 x 10^6 IU/m^2/day; therefore, no patients were enrolled at a starting dose of 2.25 x 10^6 IU/m^2/day.

Clinical tolerability of ex vivo IL-2 activated cell infusions

All patients who received subcutaneous IL-2 at less than the MTD (0.25 - 1.25 x 10^6 IU/m^2/day) received a cell dose of 4.0 x 10^7 cells/kg (Table 3). Grade I toxicities observed infrequently at this dose

level included fever and chills at time of the infusion. At the MTD of subcutaneous IL-2, 1.75×10^6 IU/m^2/day, 4 patients received a cell dose of 4.0×10^7 cells/kg (1 patient underwent one apheresis, the remaining patients two aphereses); 6 patients received 8.0×10^7 cells/kg (2 patients underwent one apheresis, the remaining 4 patients two aphereses);10 patients underwent two lymphaphereses with reinfusion of the full product. Grade I-II toxicities included wheezing (n=1), mild fever (37.1-38.0^{0}C) (n=4), chills (n=4), transient decrease in oxygen saturation to 88% during chills (n=1), and transient decrease of systolic blood pressure to 80-85 mm Hg (n=1). No patient required hospitalization. Two patients were removed from study after the first apheresis (1 for facial swelling, 1 for a bacterial infection as above). A third patient declined the second apheresis secondary to difficulties with venous access but remained on subcutaneous IL-2 for the duration of the study.

Clinical tolerability of IL-2 boluses

All 23 patients received subcutaneous IL-2 at the MTD of 1.75×10^6 IU/m^2/day. Three patients made up the first cohort, and all 3 received 42 days of subcutaneous IL-2 injections and both planned IV IL-2 boluses of 2×10^6 IU/m^2 (Table 4). Six (50%) of the 12 patients enrolled at the 4×10^6 IU/m^2 dose level received both IV boluses. Both boluses were not infused due to thrombocytopenia (n=2), Clostridium difficile infection (n=1), rash and insomnia (n=1), and neutropenia (n=1). Of the 12 patients, 8 received all 41 - 42 doses of subcutaneous IL-2; 4 patients received ≤23 days of subcutaneous IL-2. Grade I toxicities occurring at the IL-2 bolus dose of 4×10^6 IU/m^2 included fever (n=2), chills (n=3), and shortness of breath (n=1). Orthostatic hypotension occurred in 2 patients - one patient responded to intravenous normal saline, the other had spontaneous resolution of symptoms. All 8 patients enrolled at the highest dose of bolus IL-2, 6×10^6 IU/m^2, received all 42 days of subcutaneous IL-2 injections and both infusions. Grade II toxicities of fever and chills were noted in all patients during the bolus infusions. Three patients experienced a ≥10 mmHg decline in systolic blood pressure and required infusion of normal saline for blood pressure support.

Immune activation after IL-2 therapy

Laboratory tests were performed every two weeks to monitor immune activation. There was a significant increase in the total white blood cell count, peaking at day 14 and after each activated cell infusion or IL-2 bolus infusion, analogous to our previously reported findings with post-transplant subcutaneous IL-2 (4). The increase in total white blood cell count was primarily due to an increase in the number of circulating lymphocytes and eosinophils; however, the absolute number of circulating monocytes did not change with IL-2 therapy.

The lymphocyte increase included a 10-fold increase in circulating NK cells which was sustained throughout the IL-2 treatment period. Less than 2% of CD56+ cells expressed CD3 at any time point. In contrast to the increase in NK cells, the absolute number of T-cells remained fairly stable throughout IL-2 administration. Again, this was consistent with our previous findings (4).

We tested the lytic function of PBMNC obtained at study entry prior to any subcutaneous IL-2 administration, pre- and post-infusion of IL-2 activated cells, and also tested the *ex vivo* activated product against Raji and MCF-7 cells (Figure 2). Prior to subcutaneous IL-2, fresh nonactivated PBMNC had no lytic activity (<10%). *In vitro* cytotoxicity was modestly enhanced following 28 days of subcutaneous IL-2. In contrast, cytotoxicity was markedly enhanced for cells incubated overnight with IL-2 (product), and from fresh PBMNC obtained the day post-infusion of the activated cells. The lytic activity of PBMNC obtained from patients after 42 days of subcutaneous IL-2 was similar to that at 28 days. In addition, the enhancement in cytotoxicity following the second IL-2 activated cell infusion on day 42 was as potent as the first (data not shown). This suggests that the enhanced cytolytic function of the IL-2 activated cells was less than 2 weeks in duration.

The lytic function of PBMNC obtained pre-infusion and 1 day post- infusion of IV boluses of IL-2 is shown in Figure 3. Cytolytic activity after administration of subcutaneous IL-2 alone was similar to that of patients in part I of the study. Post-infusion of IL-2 boluses, cytotoxicity was markedly enhanced to a degree comparable to that seen following infusion of *ex vivo* IL-2 activated cells.

Cytokines induced by IL-2

We previously reported that subcutaneous administration of IL-2 in the post-transplant setting induced the release of soluble IL-2Rα and IFN-γ (4). In contrast, neither IL-12, TNF-α, IL1-β nor IL-6 were significantly augmented compared to baseline levels. In the current study we determined the circulating cytokine levels of IL-6, IFN-γ, TNF-α, and IL1-β in patients at day 0, day 14, and throughout their course of bolus IL-2 infusions. As shown in Figure 4, IL-2 boluses transiently increased the levels of all four cytokines, although the increase in the level of IL-1β was minimal. Peak levels of IL-6, IFN-γ, and TNF-α were noted 2 hours after each IL-2 infusion. The peak level for IL1-β occurred 24 hours after infusion; a second peak was not noted after the second IL-2 bolus. There was a dose dependent increase in IL-6 and IFN-γ. Patients who received 4-6 x 10^6 IU/m^2 had a statistically significant greater increase in peak cytokine levels of IL-6 and IFN-γ compared to patients who received 2 x 10^6 IU/m^2 of IL-2.

Clinical outcome following IL-2 immunotherapy: Matched pairs analysis

Fourteen (56%) of the 25 patients with lymphoma and 13 (59%) of the 22 patients with metastatic breast cancer who received $\geq$ 1.75 x 10^6 IU/m^2/day of subcutaneous IL2 in conjunction with at least one of the planned IL-2 activated cell infusions or IL-2 boluses were able to be matched with control patients from the ABMTR. The other 20 patients had no suitable controls identifiable. Three patients with lymphoma could be matched with 1 or 2 control patients each, and the remaining 8 patients could be matched with 3 control patients from the registry. For the 13 patients with breast cancer, one patient was matched with 1 control, 3 patients with 2 controls, and 9 patients could be matched with 3 controls from the registry. Patient characteristics are shown in Table 5. There were either very minimal or no differences between case patients and control patients for all matching characteristics. Disappointingly, we observed no difference in survival or disease-free survival between case and control patients (Table 6). The power to detect a difference in this analysis was low due to the limited patient numbers, but no suggested advantage for the IL-2 based therapy was apparent.

Discussion

Our previous study demonstrated that subcutaneous IL-2 could be safely given in the post-transplant time period, and suggested that additional *ex vivo* incubation of PBMNC in IL-2 generates NK cells with potent antitumor effects *in vivo* (4). The goal of this study was to test these observations by performing a cell dose escalation of IL-2 activated cell therapy with concomitant determination of NK cell cytotoxicity. We subsequently sought to minimize patient inconvenience as well as the cost associated with *ex vivo* incubation and reinfusion of activated cells by substitution with bolus IL-2 infusions. We established the MTD of subcutaneous IL-2 at 1.75 x 10^6 IU/m^2/day and the cell infusions were well tolerated with most patients experiencing Grade I-II toxicites. Cytotoxicity was markedly enhanced for cells incubated overnight with IL-2, and from fresh PBMNC obtained the day post-infusion of the IL-2 activated cells against both lymphoma and breast cancer targets. We subsequently demonstrated that IL-2 boluses could substitute for *ex-vivo* activated cells, with maintenance of both patient safety and the immune enhancing effects of IL-2.

Toxicities reported in a number of studies with IL-2 and IL-2 activated effectors have included mild to moderate fever, chills, rash, nausea, dyspnea, thrombocytopenia, and transient hypotension (7,10,11,13, 18,19,21,24), similar to the toxicities noted in our study. Also in agreement with other reports, side effects rapidly resolved after completion of IL-2 treatment. Although direct comparison of severity of toxicities between studies is problematic, collectively they demonstrate that IL-2 as well as autologous activated NK cells can be safely administered to patients in an inpatient or outpatient setting.

Administration of IL-2 following autologous transplantation may improve immunologic function by inducing cellular changes and indirectly by stimulating the release of cytokines (7,8). Normally, following autologous transplantation, endogenous NK cells are absent from the blood and appear no earlier than the third week (7). These cells are highly responsive to IL-2 both *in vitro* and *in vivo.* Chronic administration of low-dose IL-2 results in a lymphocytosis with expansion of NK cells without an increase in T cells, but only a modest enhancement in cytotoxic activity against tumor targets (4, 29). With increased doses of IL-2, increased percentages of PBMNCs express CD16 and CD56,

reflecting the induction of circulating NK and activated NK effector cells (13). Cytotoxicity assays demonstrate that lysis of tumor targets is significantly augmented with higher doses of IL-2 (7). In 1985, Lotze et al demonstrated that the *ex vivo* incubation of lymphocytes collected after *in vivo* IL-2 administration resulted in the generation of potent anti-tumor effector cells (30). Many investigators have since confirmed this observation, and have demonstrated as well that PBMNC obtained post-infusion of IL-2 activated cells have enhanced tumor lysis, similar to the findings in the current study. Pathological findings suggestive of skin graft-versus-host disease have also been reported (14), further supporting the immune activating effects of IL-2.

NK cells constitutively express receptors for monocyte-derived cytokines (monokines) and produce critical cytokines (including IFN-γ, TNF-α, and granulocyte-macrophage colony-stimulating factor [GM-CSF]) in response to monokine stimulation (31). In addition to their presumed role in the constitutional symptoms observed with IL-2 administration (32), theoretically cytokines could be either important mediators or suppressors of IL-2 cell activation (33-35). Therefore, several studies have attempted to define the nature of the complex cytokine cascade following IL-2 administration. Human clinical trials with IL-2 have shown that IL-6, granulocyte colony-stimulating factor (G-CSF), and GM-CSF, are induced in the blood after administration of IL-2 (36). Bonig et al studied the kinetics of IL-1β, IL-4, IL-5, IL-10, IL-12, soluble Fas ligand and GM-CSF during IL-2 therapy (37). While IL-1β, IL-4 and IL-12 were not affected, and soluble Fas ligand only mildly affected by IL-2 therapy, a consistent and early rise of IL-10, IL-5, and GM-CSF was observed. Cytokine production has also been measured in supernatants of marrow mononuclear cells cultured in IL-2 (38). TNF-α, IFN-γ, and IL-6 were produced in significant amount; in contrast, increases in IL-3, IL-7, G-CSF and GM-CSF were not detectable. In the current study, IL-2 boluses transiently increased the levels of IL-6, INF-γ, TNF-α and IL1-β, with a dose dependent increase in IL-6 and IFN-γ.

Several trials with differing eligibility and design have been performed with the aim of evaluating the efficacy of immunotherapy with IL-2 in lymphoma. Benyunes et al (10) treated 22 lymphoma patients

with IL-2 with or without lymphokine-activated killer cells after autologous transplantation. At the time of transplantation, 7 patients were in untreated or chemotherapy-sensitive first relapse, 3 were in and 12 were beyond a second clinical remission. The clinical outcomes compared favorably with institutional controls. Nagler et al conducted a phase II clinical trial involving 56 lymphoma patients with minimal residual disease following autologous stem cell transplantation utilizing a combination of subcutaneous IL-2 and INF-α in an outpatient setting and compared the results with 61 matched historical controls (16). Following hematopoietic reconstitution, patients were treated with daily subcutaneous injections of IL-2 (Chiron) at a dose of 3 to 6 x 10^6 IU/m^2/day combined with IFN-α2a (3 x 10^6 IU/day) for 5 consecutive days per week for 4 weeks. After a one month interval, a second identical course was administered. The overall and disease-free survival of patients treated with IL-2 and IFN-α were significantly higher than historical controls. The relapse rate was also significantly lower for patients who received immunotherapy. In a phase I/II study, Robinson et al treated 19 patients with lymphoma with escalating doses of 'induction' IL-2; following a 4-day rest period, maintenance IL-2 was given by continuous intravenous infusion for 10 days (13). Encouragingly, 58% of the non-Hodgkin's lymphoma patients in the phase II trial remained in clinical remission with a minimum of follow-u of 1 year. Margolin et al reported 24 patients with lymphoma who received bone marrow and/or granulocyte colony-stimulating factor (G-CSF)-mobilized autologous peripheral blood stem cells that had been exposed to IL-2 for 24 hours *ex vivo* (20). Patients subsequently received IL-2 by low-dose continuous IV infusion until hematologic reconstitution and then by intermediate-dose continuous IV infusion for six 2-week maintenance cycles given at 1-month intervals. Among the 24 lymphoma patients, nine were in continuous complete remission from 18-48 months, and 15 had died (12 due to relapse and 3 to therapy related toxicities).

Other investigators have attempted to determine if IL-2 therapy is efficacious in patients with metastatic breast cancer with conflicting results. Gravis et al noted no clinical beneficial effect for 21 advanced heavily pretreated breast cancer patients treated with either IV high-dose IL-2 or subcutaneous low dose IL-2 following high dose chemotherapy and autologous stem cell transplantation (23). Toh et al

reported a prospective phase II trial of 33 patients with chemosensitive metastatic breast cancer who underwent transplantation with autologous peripheral blood stem cells cultured in IL-2 for 24 hours as adoptive immunotherapy (24). Low-dose IL-2 was subsequently administered from day 0 - 4 and/or 7 -11, 14 -18, 25 - 29, then 5 days per month for 11 months. Compared to historical controls group, the Kaplan-Meier estimated 2 year progression-free survival (PFS) was 35% for IL-2 treated patients, compared with 17% in the control arm (P = 0.73) and the estimated 2 year survival was 78%, compared with 61% in the control arm (P = 0.22).

In the current study we sought to determine if subcutaneous IL-2 and IL-2 activated cells/IL-2 could improve clinical outcomes of survival and disease-free survival. There was no apparent advantage to IL-2 based immunotherapy, but, as the power to detect a difference in this matched-pairs analysis was low due to limited patient numbers, no conclusions can be drawn regarding clinical efficacy. Enhancement of the potency or duration of IL-2 activated NK cytotoxicity may require utilization of highly purified NK cells or NK cell subsets, a combination of NK cells with tumor-reactive monoclonal antibodies to induce effective antibody dependent cellular cytotoxicity (39), combinations of NK cell immunotherapy with chemotherapy or other cytokines such as IL-12 (40), or augmentation by NK cell inhibitory receptor blockade (41). Indeed, as malignant cells may have multiple mechanisms by which they escape from immune control, more than one immunotherapeutic intervention may be required. Further study is needed to take advantage of the immunotherapeutic potential of IL-2 activated NK cells.

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References

1. Weisdorf DJ, Verfaillie CM, Miller WJ, et al. Autologous bone marrow versus non-moblilized peripheral blood stem cell transplantation for lymphoid malignancies: a prospective, comparative trial. *Am J Hematol.* 1997; 54:202-208.
2. Antman KH, Rowlings PA, Vaughan WP, et al. High-dose chemotherapy with autologous hematopoietic stem-cell support for breast cancer in North America. *J Clin Oncol.* 1997;15:1870-1879.
3. Verma UN, Areman E, Dickerson SA, Kotula PL, Sacher R, Maxumder A. Interleukin-2 activation of chemotherapy and growth factor-mobilized peripheral blood stem cells for generation of cytotoxic effectors. *Bone Marrow Transplantation.* 1995;15:199-206.
4. Miller JS, Tessmer-Tuck J, Pierson BA, et al. Low dose subcutaneous interleukin-2 after autologous transplantation generates sustained in vivo natural killer cell activity. *Biol Blood Marrow Transplant.* 1997; 3:34-44.
5. Gottlieb DJ, Brenner MK, Heslop HE, et al. A phase I clinical trial of recombinant interleukin 2 following high dose chemo-radiotherapy for haematological malignancy: Applicability to the elimination of minimal residual disease. *Br J Cancer.* 1989;60:610-615.
6. Blaise D, Olive D, Stoppa AM, et al. Hematologic and immunologic effects of the systemic administration of recombinant interleukin-2 after autologous bone marrow transplantation. *Blood.* 1990; 76:1092-1097.
7. Higuchi CM, Thompson JA, Petersen FB, Buckner CD, Fefer A. Toxicity and immunomodulatory effects of interleukin-2 after autologous bone marrow transplantation for hematologic malignancies. *Blood.* 1991; 77:2561-2568.
8. Soiffer RJ, Murray C, Cochran K, et al. Clinical and immunologic effects of prolonged infusion of low dose recombinant interleukin-2 after autologous and T-cell depleted allogeneic bone marrow transplantation. *Blood.* 1992; 79:517-526.

9. Weisdorf DJ, Anderson PM, Blazar BR, Uckun FM, Kersey JH, Ramsay NK. Interleukin-2 immediately after autologous bone marrow transplantation for acute lymphoblastic leukemia: a phase I study. *Transplantation.* 1993; 55:61-66.

10. Benyunes MC, Higuchi C, York A, et al. Immunotherapy with interleukin-2 with or without lymphokine-activated killer cells after autologous bone marrow transplantation for malignant lymphoma: a feasibility trial. *Bone Marrow Transplant.* 1995; 16:283-288.

11. Lister J, Rybka WB, Donnenberg AD, et al. Autologous peripheral blood stem cell transplantation and adoptive immunotherapy with activated natural killer cells in the immediate posttransplant period. *Clin Cancer Res.* 1995; 6:607-614.

12. Mazumder A. Experimental evidence of interleukin-2 activity in bone marrow transplantation. *Cancer J Sci Am.* 1997; 3:Suppl1:S37-42.

13. Robinson N, Benyunes MC, Thompson JA, et al. Interleukin-2 after autologous stem cell translantation for hematologic malignancy: a phase I/II study. *Bone Marrow Transplant.* 1997; 19:435-442.

14. Meehan KR, Verma UN, Cahill R, et al. Interleukin-2-activated hematopoietic stem cell transplantation for breast cancer: investigation of dose level with clinical correlates. *Bone Marrow Transplant.* 1997; 20:643-651.

15. Meloni G, Vignetti M , Pogliani E, et al. Interleukin-2 therapy in relapsed acute myelogenous leukemia. *Cancer J Sci Am.* 1997; 3:S1:S43-47.

16. Nagler A, Ackerstein A, Or R, Naparstek E, Slavin S. Immunotherapy with recombinant human interleukin-2 and recombinant interferon-alpha in lymphoma patients post-autologous marrow or stem cell transplantation. *Blood.* 1997; 89:3951-3959.

17. Blaise D, Attal M, Pico JL, et al. The use of a sequential high dose recombinant interleukin 2 regimen after autologous bone marrow transplantation does not improve the disease free survival of patients with acute leukemia transplanted in first complete remission. *Leuk Lymphoma.* 1997; 25:469-478.

18. Van Besien K, Margolin K, Champlin R, Forman S. Activity of interleukin-2 in non-Hogkin's lymphoma following transplantation of interleukin-2-activated autologous bone marrow or stem cells. *Cancer J Sci Am.* 1997; 3 Suppl1:S54-58.

19. Meehan KR, Arun B, Gehan EA, et al. Immunotherapy with interleukin-2 and alpha-interferon after IL-2 activated hematopoietic stem cell transplantation for breast cancer. *Bone Marrow Transplant.* 1999; 23:667-673.

20. Margolin KA, Van Besien K, Wright C, et al. Interleukin-2-activated autologous bone marrow and peripheral blood stem cells in the treatment of acute leukemia and lymphoma. *Biol Blood Marrow Transplant.* 1999; 5:36-45.

21. DeMagalhaes-Silverman M, Donnenberg A, Lembersky B, et al. Posttransplant adoptive immunotherapy with activated natural killer cells in patients with metastatic breast cancer. *J Immunother.* 1999; 23:154-160.

22. Blaise D, Attal M, Reiffers J, et al. Randomized study of recombinant interleukin-2 after autologous bone marrow transplantation for acute leukemia in first complete remission. *Eur Cytokine Netw.* 2000;11:91-98.

23. Gravis G, Viens P, Vey N, et al. Pilot study of immunotherapy with interleukin-2 after autologous stem cell transplantation in advanced breast cancers. *Anticancer Res.* 2000; 20:3987-3991.

24. Toh HC, McAfee SL, Sackstein R, et al. High-dose cyclophosphamide + carboplatin and interleukin-2 (IL-2) activated autologous stem cell transplantation followed by maintenance IL-2 therapy in metastatic breast carcinoma - a phase II study. *Bone Marrow Transplant.* 2000; 25:19-24.

25. Toren A, Nagler A, Rozenfeld-Granot G, et al. Amplification of immunological functions by subcutaneous injection of intermediate-high dose interleukin-2 for 2 years after autologous stem cell transplantation in children with stage IV neuroblastoma. *Transplantation.* 2000; 70:1100-1104.

26. Burns LJ, Weisdorf DJ, DeFor TE, et al. Enhancement of the anti-tumor activity of a peripheral blood progenitor cell graft by mobilization with interleukin 2 plus granulocyte colony-stimulating factor in patients with advanced breast cancer. *Exp Hematol.* 2000; 28:96-103.

27. Meropol NJ, Porter M, Blumenson LE, et al. Daily subcutaneous injection of low-dose interleukin 2 expands natural killer cells in vivo without significant toxicity. *Clin Cancer Res.* 1996; 2:669-677.

28. Gail MH, Lubin JH, Rubenstein L.V. Liklihood calculations for matched case-control studies and survival studies with tied death times. *Biometrika.* 1981; 68:703-707.

29. Soiffer RJ, Murray C, Cochran K, et al. Clinical and immunologic effects of prolonged infusion of low-dose recombinant interleukin-2 after autologous and R-cell-depleted allogeneic bone marrow transplantation. *Blood.* 1992; 79:517-526.

30. Lotze MT, Matory YL, Ettinghausen SE, et al. In vivo administration of purified human interleukin 2: half life, immunologic effects, and expansion of peripheral lymphoid cells in vivo with recombinant rIL-2. *J Immunol* 1985;135:2865-2875.

31. Cooper MA, Fehniger TA, Turner SC, et al. Human natural killer cells: a unique innate immunoregulatory role for the $CD56^{bright}$ subset. *Blood.* 2001; 97:3146-3151.

32. Gemlo BT, Palladino MA, Jaffe HS, Expevik TP, Rayner AA. Circulating cytokines in patients with metastatic cancer treated with recombinant interleukin 2 and lymphokine-activated killer cells. *Cancer Res.* 1988; 48:5864-5867.

33. Holter W, Goldman CK, Casabo L, Nelson DL, Greene WC, Waldmann TA. Expression of functional IL-2 receptors by lipopolysaccharide and interferon-gamma stimulated human monocytes. *J Immunol.* 1987; 138:2917-2922.

34. Greenbaum LA, Horowitz JB, Woods A, Pasqualini T, Reich EP, Bottomly K. Autocrine growth of CD4+ T cells. Differential effects of IL-1 on helper and inflammatory T cells. *J Immunol.* 1988; 140:1555-1560.

35. Mills CD. Molecular basis of "suppressor" macrophages: arginine metabolism via the nitric oxide synthesis pathway. *J Immunol.* 1991; 46:2719-

36. Tritarelli E, Rocca E, Testa U, et al. Adoptive immunotherapy with high-dose interleukin-2: Kinetics of circulating progenitors correlate with interleukin-6, granulocyte colony-stimulating factor level. *Blood.* 1991; 77:741-749.

37. Bonig H, Laws HG, Wundes A, et al. In vivo cytokine responses to interleukin-2 immunotherapy after autologous stem cell transplantation in children with solid tumors. *Bone Marrow Transplant.* 2000; 26:91-96.

38. Klinegmann HG, Neerunjun J, Schwulera U, Ziltener HJ. Culture of normal and leukemic bone marrow in interleukin-2: analysis of cell activation, cell proliferation, and cytokine production. *Leukemia.* 1993; 7:1389-1393.

39. Sondel PM, Hank JA. Combination therapy with interleukin-2 and antitumor monoclonal antibodies. *Cancer J Sci Am Suppl.* 1997; 1:S121-127.

40. Wigginton JM, Komschlies KL, Back TC, Franco JL, Brunda MJ, Wiltrout RH. Administration of IL-12 with pulse interleukin 2 and the rapid and complete eradication of murine renal carcinoma. *J Natl Cancer Inst.* 1996; 88:38-43.

41. Koh CY, Blazar BR, George T, et al. Augmentation of antitumor effects by NK cell inhibitory receptor blockade in vitro and in vivo. *Blood.* 2001; 97:3132-3137.

Table 1. *Patient characteristics*

	IL-2 Treatment	
	Part I	Part II
	IL-2 + IL-2 activated cells	IL-2 + IL2 boluses
Number of patients	34	23
Age in years, median (range)	46 (19-62)	43 (27-60)
Gender		
Male	14	4
Female	20	19
Disease		
Non-Hodgkin's Lymphoma	15	5
Follicular	8	0
Diffuse large cell	7	5
Hodgkin's Disease	5	4
Metastatic breast cancer	14	14

Table 2. *Subcutaneous IL-2 dose escalation*

Dose of IL-2 ($\times 10^6$ IU/m^2/day)	Enrolled	Dose escalated day 14	Received ≥ 95% planned doses
0.25	3	3	2
0.75	3	2	3
1.25	3	2	3
1.75	25	15	18 (72%)
2.25	0		

Table 3. *Aphereses and clinical tolerability of IL-2 activated cells*

Dose of IL-2 ($\times 10^6$ IU/m^2/day)	Enrolled	No. aphereses		Cell dose infused			Toxicity of IL-2 activated cells
		One	Two	4.0 ($\times 10^7$/kg)	8.0 ($\times 10^7$/kg)	full product	
0.25	3	1	1	2	0	0	Grade I fever, chills
0.75	3	0	3	3	0	0	
1.25	3	0	3	3	0	0	
1.75	25	3	17	4	6	10	Grade I-II wheezing, fever, chills, hypoxia, hypotension

Table 4. *Clinical tolerability of IV boluses of IL-2*

Dose of IL-2 ($\times 10^6$ IU/m^2)	Enrolled	Received both boluses	Toxicity of IL-2 boluses
2.0	3	3	None
4.0	12	6	Grade I fever, chills, or shortness of breath Hypotension requiring therapy
6.0	8	8	Grade II fever, chills Hypotension requiring therapy

Table 5. *Patient characteristics in matched pairs analysis**

	Lymphoma			Breast Cancer	
	# (%) Case	# (%) Controls		# (%) Case	# (%) Controls
Disease					
NHL	8 (57%)	20 (61%)	Metastatic	13 (100%)	34 (100%)
HD	6 (43%)	13 (39%)			
Age at Transplant					
Median (range)	37 (27-62)	42 (25-63)		42 (35-57)	42 (31-57)
Stem Cell Source					
PBSC	13 (93%)	32(97%)		12 (92%)	29 (85%)
Marrow+PBSC	1 (7%)	1 (3%)		1 (8%)	4 (12%)
Marrow	0	0		0	1 (3%)
Conditioning regimen					
TBI	2 (14%)	4 (12%)		0	0
Non-TBI	12 (86%)	28 (85%)		13 (100%)	34 (100%)
Unknown	0	1 (3%)			
			Visceral metastases		
			No	12 (92%)	31 (91%)
			Yes	1 (8%)	3 (9%)
			ER and PR		
			Positive	7 (54%)	17 (50%)
			Negative	6 (46%)	17 (50%)

*Matching characteristics also included disease histology, disease status at transplant, year of transplant (within 5 years), age at transplant (within 5 years), and time from diagnosis to transplant (within 1 year). NHL indicates non-Hodgkin's lymphoma; HD, Hodgkin's disease; peripheral blood stem cell; TBI, total body irradiation; ER, estrogen receptor; PR, progesterone receptor

Table 6. *Relative risk of death or relapse of patients in matched pairs analysis*

Group	N	Death Relative Risk (95% CI)	Death P value	Relapse Relative Risk (95% CI)	Relapse P value
Lymphoma					
Case	14	0.4 (.1-2.1)	.29	1.1 (.5-2.8)	.79
Control	33	1.0		1.0	
Breast Cancer					
Case	13	0.3 (.1-1.5)	.14	1.4 (.6-3.2)	.44
Control	34	1.0		1.0	

Shown is the relative risk (and 95% confidence interval) of death or relapse post-transplant for IL-2 treated cases compared to matched controls as determined by stratified matched pair Cox regressions.

Figure Legends

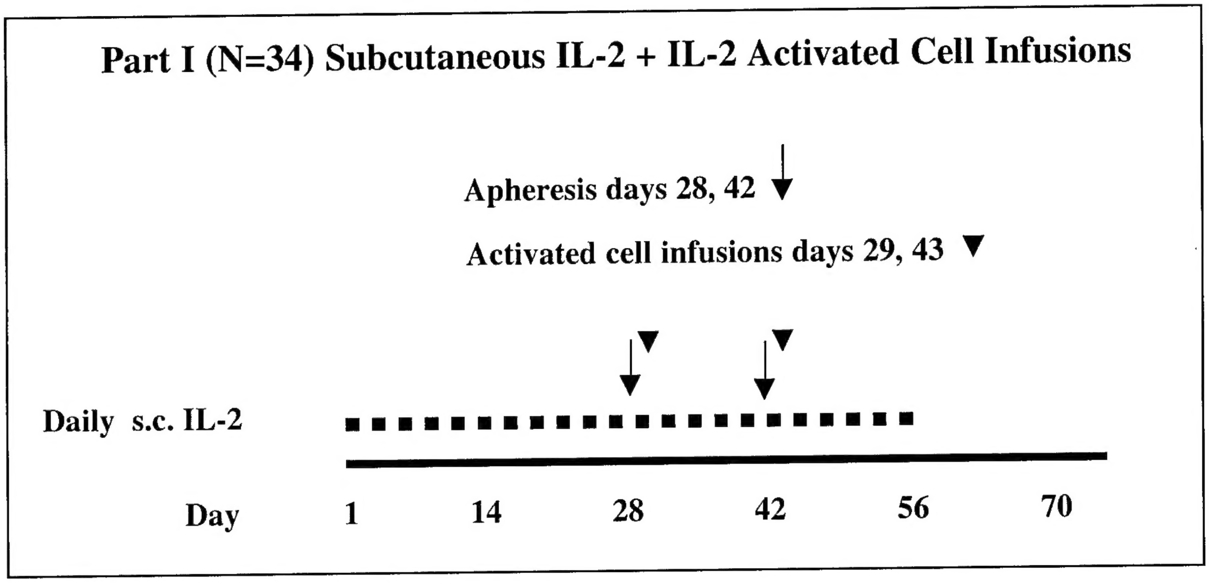
Figure 1. Schemas for part I and part II of the study.

Figure 2. *Ex vivo* IL-2 activated cell infusions enhance NK cell lytic activity against Raji and MCF-7 targets. Peripheral blood mononuclear cells (PBMNC) from patients receiving 1.75 x 10^6 IU/m^2/day of subcutaneous IL-2 were studied before and after starting post-transplant immunotherapy in a 4-hour chromium release cytotoxicity assay against Raji (left panel), MCF-7 (right panel) and K562 (data not shown) targets. Patient PBMNC were tested prior to study entry (closed squares, n=16 for Raji, n=10 for MCF-7), after 28 days of subcutaneous IL-2 alone (open circles, n=18 for Raji, n=18 for MCF-7) and 24 hours after *ex vivo* IL-2 activated NK cell infusions (closed circles, n=11 for Raji, n=15 for MCF-7). The activated NK cell infusion product (closed triangles, n=13 for Raji, n=15 for MCF-7) exhibited potent lytic activity against both targets. Lysis of both targets was significantly greater after activated cell infusions compared to the PBMNC tested immediately prior to cell infusions.

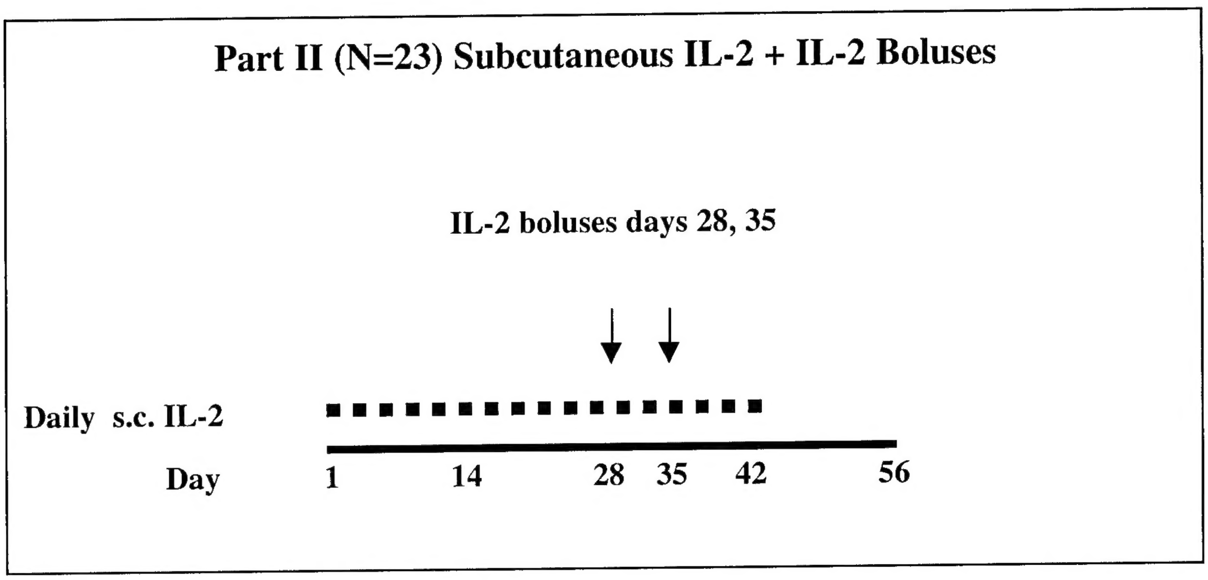
Figure 3. Intravenous IL-2 infusions can replace activated cell infusions to enhance lytic activity. PBMNC from patients receiving subcutaneous IL-2 (1.75 x 10^6 IU/m^2/day) and IL-2 infusions were studied as described in Figure 2 against Raji (left panel), MCF-7 (right panel) and K562 (data not shown) targets. Patient PBMNC after 28 days of subcutaneous IL-2 alone (open circles, n=9 for both targets) were compared to PBMNC 24 hours after a 2 hour infusion of 4 - 6 IU/m^2 intravenous IL-2 (closed circles, n=9 for both targets). Lysis of both targets was significantly greater after IL-2 infusions compared to the PBMNC tested immediately prior to infusions.

Figure 4. Intravenous IL-2 infusions induce a secondary cytokine cascade. Serum was obtained from patients receiving subcutaneous IL-2 (1.75 x 10^6 IU/m^2/day) and IL-2 infusions (4 - 6 IU/m^2/day) on days 28 and 35 as indicated by the arrows on the X-axis. IFN-γ, TNF-α, IL-6 and IL-1β were analyzed by ELISA from serum samples collected at the time points listed. The arrow on the Y-axis

indicates the cytokine level found for > 95% of healthy individuals. Each point represents the mean ± S.E.M. of paired serum samples from 5 treated patients.

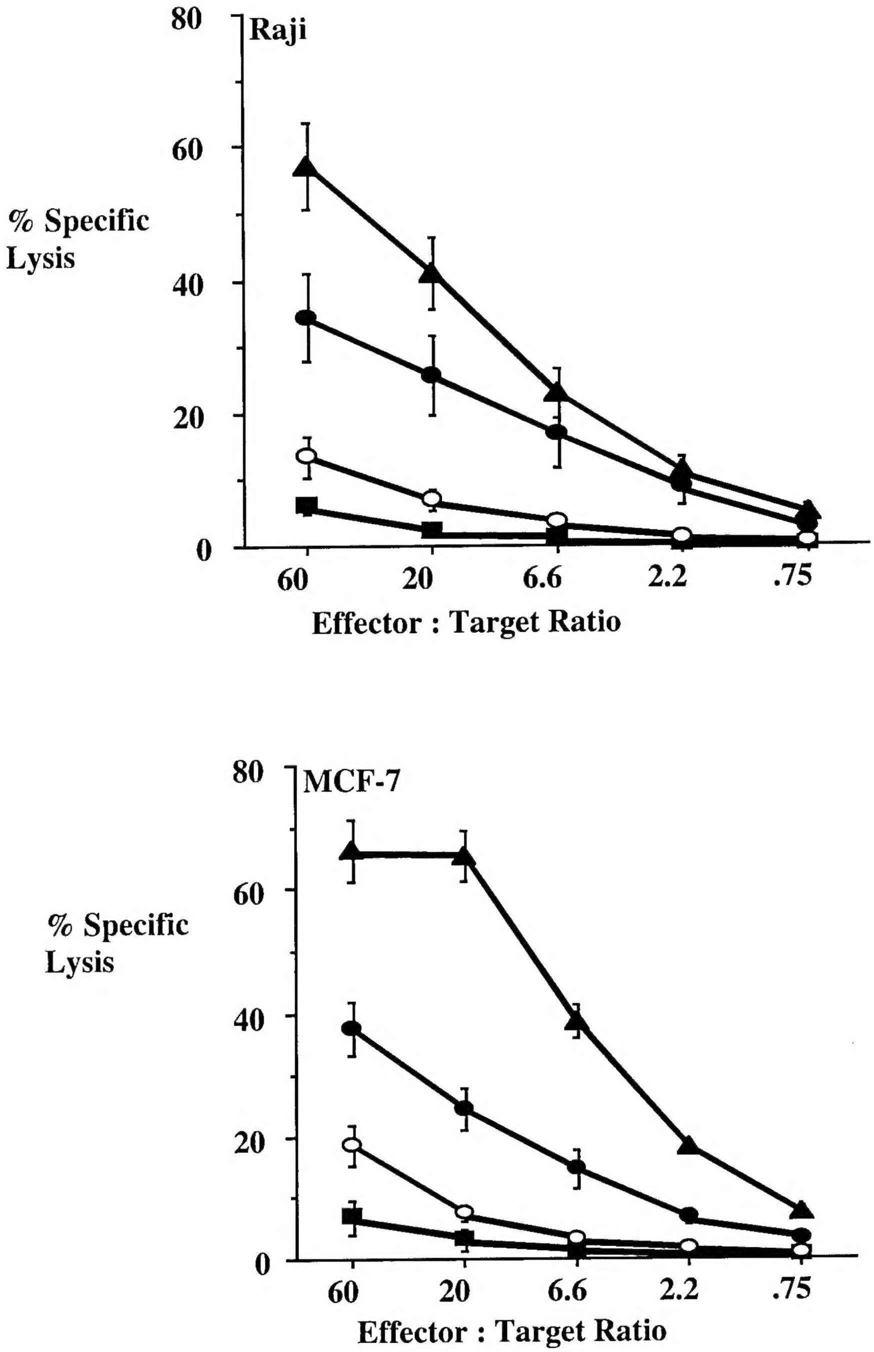


Part I (N=34) Subcutaneous IL-2 + IL-2 Activated Cell Infusions
Apheresis days 28, 42
Activated cell infusions days 29, 43
Daily s.c. IL-2
Day
1
14
28
42
56
70



Part II (N=23) Subcutaneous IL-2 + IL-2 Boluses
IL-2 boluses days 28, 35
Daily s.c. IL-2
Day
1
14
28
35
42
56

Figure 1


80
60
40
20
0
Raji
% Specific
Lysis
60
20
6.6
2.2
.75
Effector : Target Ratio
80
60
40
20
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MCF-7
% Specific
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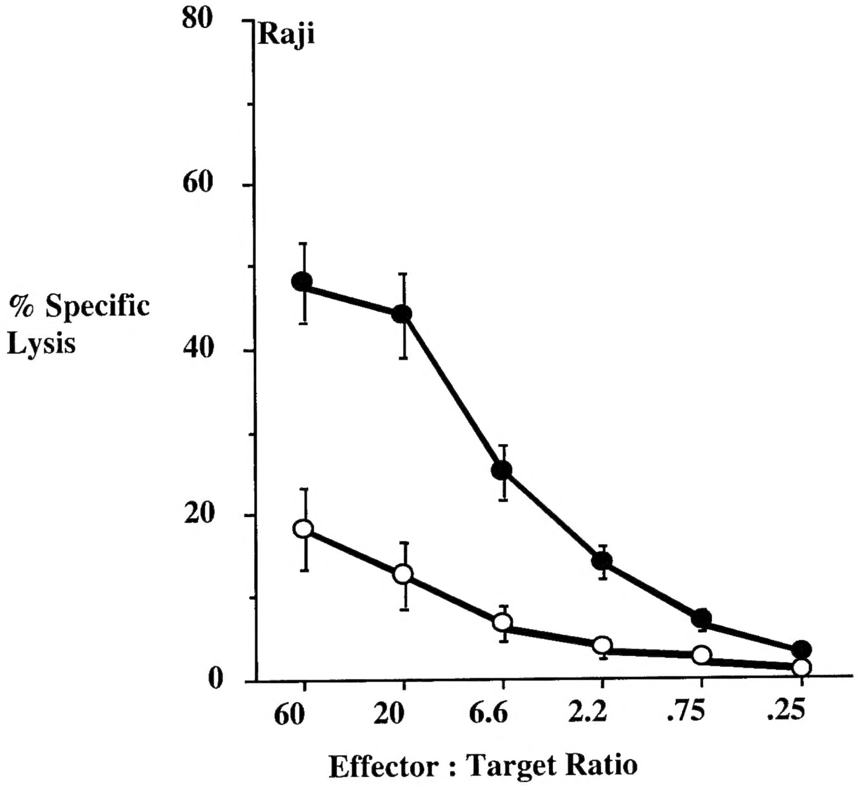
.75
Effector : Target Ratio

Figure 2


Raji
80
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Effector : Target Ratio

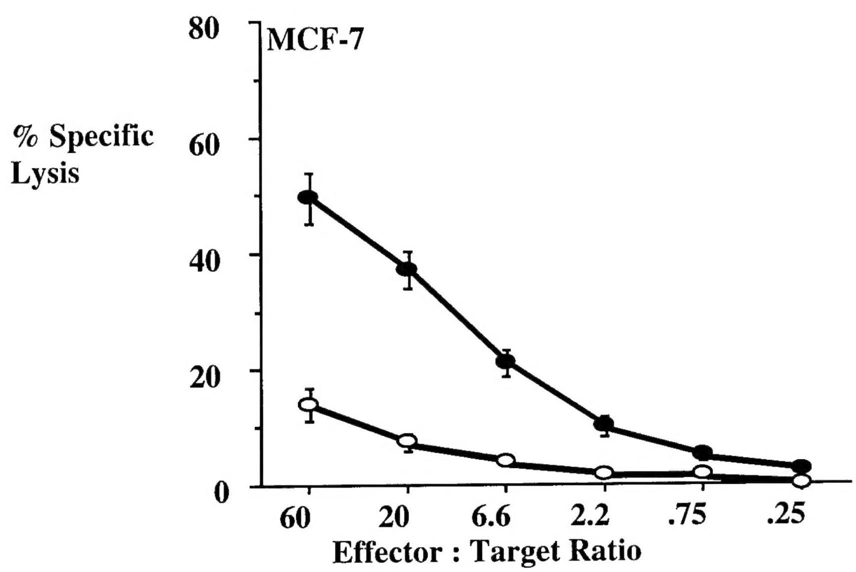
MCF-7
80
60
40
20
0
% Specific Lysis
60
20
6.6
2.2
.75
.25
Effector : Target Ratio

Figure 3

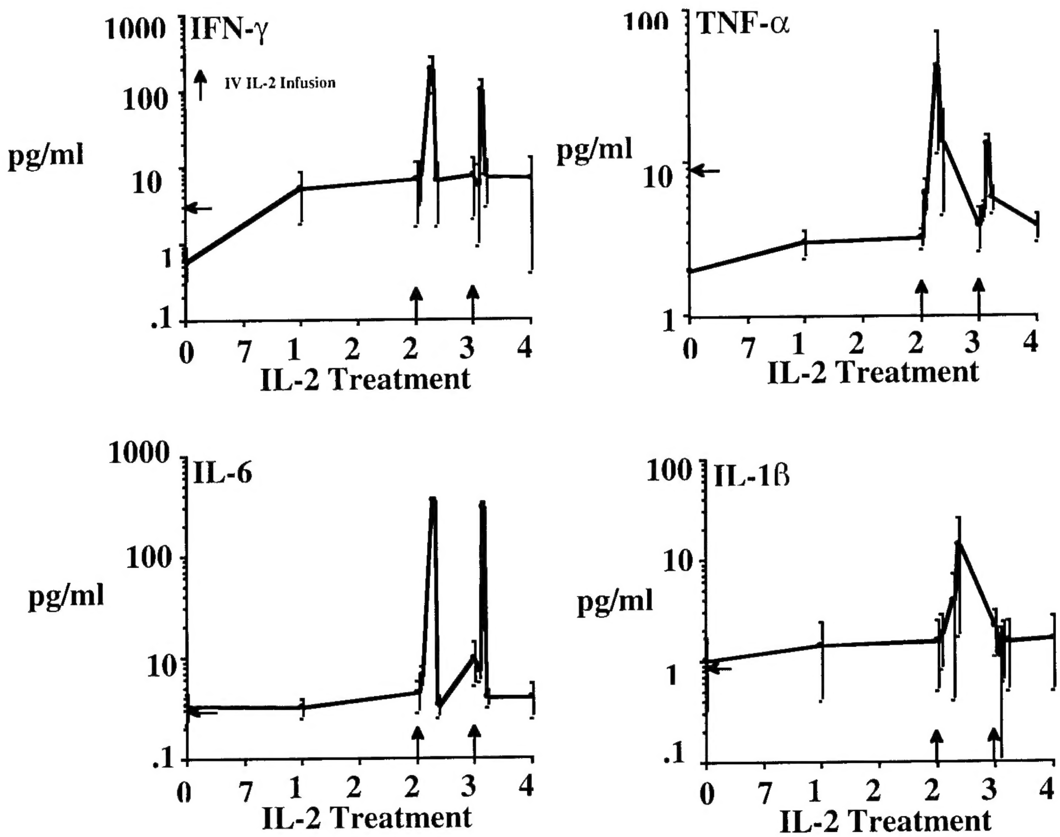
IFN-γ
IV IL-2 Infusion
1000
100
10
1
.1
pg/ml
0 7 1 2 2 3 4
IL-2 Treatment
TNF-α
100
10
1
pg/ml
0 7 1 2 2 3 4
IL-2 Treatment
IL-6
1000
100
10
.1
pg/ml
0 7 1 2 2 3 4
IL-2 Treatment
IL-1β
100
10
1
.1
pg/ml
0 7 1 2 2 3 4
IL-2 Treatment

Figure 4